

Abstract

INVESTIGATING THE ROLE OF POXVIRUS VIRULENCE GENES A35 AND O1L
IN THE VIRUS LIFE CYCLE

By

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Poxviruses, some of the largest viruses in existence, have a great impact on the human and animal world due to their ability to infect a broad assortment of organisms and cause significant disease. Today, poxvirus infections remain a danger to human health, as natural and potential bioterrorism threats. *Vaccinia virus* (VACV), the species of poxvirus used in smallpox vaccines, is the most studied poxvirus, but there is still much to learn in regards to its virulence factors and their role in the virus life cycle. Our laboratory has identified two VACV genes/proteins that play an important role in virulence, A35 and O1L, which we hypothesized were immunoregulatory, so their effects on host immune responses were assessed. We found that the A35 protein inhibits anti-viral antibody production and cytokine responses by T lymphocytes *in vivo*. However, there was no evidence to suggest that A35 inhibits recall antigen presentation by infected BMDC *in vitro*. There were also no A35 effects observed on VACV cell killing, replication, or integrin expression for bone marrow dendritic cells (BMDC), which were used as antigen presenting cells (APC). When looking at the function of O1L, we did not find an effect of O1L on anti-viral antibody production or T cell response, so the O1L effects on replication and spread, cell killing, integrin expression, cytokine production, and innate immunity were also

measured. In each of these cases, the O1L deletion mutant (O1LDel) had a similar phenotype to the wild type virus. We did observe that plaques formed by the O1LDel virus appeared smaller in some cases compared to wild type plaques, which was due to reduced cell clearance in the center of the O1LDel plaques. However, the biological relevance of this finding is unclear at this time. The fact that the VACV O1L gene encodes a large protein that is conserved in mammalian tropic poxviruses with 92-100% homology supports that the gene performs an important function in the poxvirus life cycle. Our laboratory has shown that both A35 and O1L deletion viral mutants make safer vaccine alternatives against poxviruses. Understanding how poxviruses turn off immune responses will aid in our understanding of viral pathogenesis and support anti-viral drug design, improve vaccines, and may allow us to mimic poxvirus immunosuppression to control autoimmune diseases.

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DEDICATION

I would like to dedicate this to my family and friends, especially to my mom who was my greatest advocate and to my fiancé for his support and encouragement. I could not have been a successful graduate student without them.

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TABLE OF CONTENTS

LIST OF FIGURES.....	viii
LIST OF ABBREVIATIONS.....	x
CHAPTER 1: INTRODUCTION	1
Poxvirus Threats	1
Smallpox Vaccines and Treatments.....	3
Medical Applications of Poxviruses	6
Viral Virulence.....	7
A35 and O1L Poxvirus Genes.....	8
Characterization of A35.....	8
Characterization of O1L.....	11
CHAPTER 2: MATERIALS AND METHODS	15
Cells and Virus.....	15
One Step Growth Curve.....	16
Viral Plaque Assay.....	16
Antigen Presentation	17
Antigen Presentation with Splenocytes only.....	18
Antigen Presentation to Transgenic T Cells.....	18
Metabolism (MTS) Assay	18
CTL IL-2 Bioassay	19
Nitric Oxide (NO) Assay	19
Anti-viral Antibody ELISA.....	20
Flow Cytometry	21

Cell Killing.....	22
Cytokine Bead Assay.....	22
Worm Hemolymph Assay.....	23
Statistical Analysis.....	24
CHAPTER 3: RESULTS.....	25
VACV A35 Gene	25
VACV Killing and Replication in BMDC.....	25
Effects of A35 on Antigen Presentation and T Cell Response.....	26
A35 and Anti-viral Antibody Production.....	27
Effect of A35 on Integrin Expression.....	28
VACV O1L Gene.....	29
Role of O1L in Antigen Presentation and T Cell Response.....	29
Antigen Presentation to Transgenic T Cells.....	32
O1L and Anti-viral Antibody Production.....	33
O1L and Cell Killing.....	34
Effect of O1L on Integrin Expression.....	35
Cytokine Production.....	36
Effect of O1L on Innate Immunity.....	37
CHAPTER 4 : FIGURES	38
CHAPTER 5 : DISCUSSION.....	63
REFERENCES.....	72
APPENDIX -Animal Use Protocol Letter.....	81

LIST OF FIGURES

Figure 1: A35 Cell killing in BMDC.....	38
Figure 2: One Step Growth Curve BALB/c and C57BL/6 BMDC.....	39
Figure 3: A35 Antigen Presentation (IL-2).....	40
Figure 4: A35 Antigen Presentation (NO).....	41
Figure 5: A35 ELISA Weeks 1-4.....	42
Figure 6: A35 ELISA (line of best fit analysis).....	43
Figure 7: A35 Integrin Flow Cytometry.....	44
Figure 8: O1L Antigen Presentation (IL-2).....	45
Figure 9: O1L Antigen Presentation 10,000 BMDC (IL-2).....	46
Figure 10: O1L Antigen Presentation #2 (IL-2).....	47
Figure 11: O1L Antigen Presentation 50,000 BMDC or MOI=100 (IL-2).....	48
Figure 12: O1L Antigen Presentation with Splenocytes only (IL-2).....	49
Figure 13: O1L Antigen Presentation with Splenocytes only (NO).....	50
Figure 14: O1L Antigen Presentation to Transgenic T Cells (IL-2).....	51
Figure 15: O1L ELISA Weeks 1-4.....	52
Figure 16: O1L ELISA Repeat Week 3.....	53
Figure 17: O1L ELISA BALB/c Week 1.....	54
Figure 18: O1L ELISA #2 Weeks 2-5.....	55
Figure 19: O1L Replication and Spread.....	56
Figure 20: O1L Replication and Spread (higher magnification).....	57
Figure 21: Cell killing in BMDC.....	58
Figure 22: Cell killing in CTLL and Splenocytes.....	59

Figure 23: O1L Integrin Flow Cytometry.....	60
Figure 24: Cytokine Bead Assay.....	61
Figure 25: Worm Hemolymph Assay.....	62

LIST OF ABBREVIATIONS

A35Del - A35 deletion mutant virus

APC - antigen presenting cell

BMDC - bone marrow dendritic cells

BS-C-1 - African green monkey kidney cell line

CDC - Centers for Disease Control

CDV - Cidofovir

CLIP - class II associated invariant chain peptide

CTLL - cytotoxic T lymphocyte line cells

CVA - Chorioallantois Vaccinia Ankara virus

CVA-O1LDel - CVA O1L deletion mutant virus

egfp - enhanced green fluorescent protein

ELISA - enzyme-linked immunosorbent assay

FBS - fetal bovine serum

FDA - U.S. Food and Drug Administration

GMSCF - granulocyte macrophage colony stimulating factor

HLA-DM - human leukocyte antigen DM protein

hpi - hours post infection

ICAM-1 - intercellular adhesion molecule 1

IFN - interferon

IFN- γ - interferon gamma

IL-2 - interleukin 2

IL-4 - interleukin 4

IL-6 - interleukin 6

IL-10 - interleukin 10

IL-17 - interleukin 17A

LFA-1 - lymphocyte function-associated antigen 1

MEM - minimum essential media

MHC - major histocompatibility complex

MOG - myelin oligodendrocyte glycoprotein

MOI - multiplicity of infection

MTS - 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt

MVA - Modified Vaccinia Ankara virus

NFM - neurofilament medium polypeptide

NO - nitric oxide

O/N - overnight

O1LDe1 - O1L deletion mutant virus

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PEC - peritoneal exudate cells

Pfu - plaque forming units

RPMI - Roswell Park Memorial Institute medium

RT - room temperature

TNF - tumor necrosis factor

UN - uninfected cells

VACV - Vaccinia virus

VCAM-1 - vascular cell adhesion protein 1

VLA-4 - very late antigen 4

WR - Western Reserve virus (wild type)

CHAPTER 1 : INTRODUCTION

1) Poxvirus Threats

Poxviruses are notorious for the lethal mark they have made on human history. The most infamous member of the *Poxviridae* family is Variola virus, which is the Orthopoxvirus responsible for smallpox. Smallpox was an intensely feared disease known for high mortality rates, rapid transmission, and serious health problems for survivors (Voigt, 2016). Infection began with an incubation period of 8-14 days, followed by symptoms of fever, shivering, and joint pain, which culminated in a rash with blisters and pustules (Théves, Biagini, & Crubézy, 2014). In the 20th century, approximately 500 million people died of infection until 1980 when smallpox was successfully eradicated worldwide due to an aggressive vaccination program using another Orthopoxvirus, Vaccinia virus (VACV) (Mahalingam, Damon, & Lidbury, 2004).

Smallpox became the first human pathogen eradicated in history, but poxviruses remain a threat today. They infect a large variety of animals including insects, reptiles, birds, and mammals. In recent years, a number of poxviruses have caused outbreaks in humans and animals worldwide. Monkeypox virus is endemic to Africa and thought to be the most dangerous extant human infecting poxvirus. Smallpox and smallpox-related poxviruses like Monkeypox are considered bioterrorism concerns and are listed as category A pathogens, meaning they are easily transmitted from person to person and result in high mortality rates (Reidel, 2005). The symptoms of monkeypox in humans closely resemble smallpox and may have been misdiagnosed as smallpox prior to 1970 when the first human infections with monkeypox were confirmed (Reynolds & Damon, 2012). Monkeypox mainly affects people living in the Congo Basin, specifically the Democratic Republic of Congo, which recent epidemiologic studies suggest are experiencing a reemergence of the virus (Reynolds & Damon,

2012). Monkeypox is able to infect humans and has animal reservoirs, making it more difficult to eradicate. An outbreak of monkeypox occurred in the U.S. in 2003 and was traced back to a shipment of live animals exported from West Africa (Chen et al., 2005; Reynolds & Damon, 2012). Several other poxviruses known to infect humans include cowpox in Europe and Asia (Lewis-Jones, 2004), buffalopox in India (Kolhapure et al., 1997), and Cantagalo in South America (Damaso, Esposito, Condit, & Moussatche, 2000; Oliveira et al., 2013; Shchelkunov, 2013). In the U.S., the most prevalent poxvirus is Molluscum contagiosum, which commonly infects children and immunocompromised patients, accounts for approximately 300,000 doctor visits a year, and is an emerging sexually transmitted disease (Molino, Fleischer, & Feldman, 2004). A 2009 survey of 100 pediatric dermatologists revealed approximately two-thirds of the providers reported seeing 1-10 cases of Molluscum contagiosum per week (Shisler, 2015).

With the exception of Molluscum contagiosum and smallpox which only infect humans, the majority of human poxvirus infections are zoonotic and transferred through close contact with infected animals. Cowpox in particular is a threat for exotic zoo animals and the humans who interact with them. It has the largest and most complete genome of all Orthopoxviruses, giving it a broad host range; however elephants and exotic felids remain the most commonly infected groups (Kurth, 2009). There were seasonal outbreaks of cowpox in cheetahs at the same safari park in Denmark between 2010 and 2014 (Stagegaard, 2017), as well as occurrences reported in other exotic animals including mongooses, rhinoceroses, monkeys, red pandas, and llamas (Kurth, 2009). In 2002, an outbreak of cowpox in a colony of 80 New World monkeys resulted in a 40% fatality rate (Mätz-Rensing et al., 2006). This is the most significant cowpox outbreak to date because it suggests the virus could mutate into being highly transmissible and pathogenic in humans as well. Similar to monkeypox, cowpox virus is transmitted by rodents

and other animals. In 2009, there were 33 human cases of cowpox diagnosed in Germany and France that were ultimately attributed to contact with infected pet rats (Shimshony, 2009).

Reminiscent of the monkeypox outbreak in 2003 in the U.S., this incident suggests the exotic pet trade may be a factor in the spread of poxviruses. Another circumstance affecting the occurrence of poxvirus infections is the safety and handling protocols for research laboratories. Although cowpox infections have been limited to Europe and Asia, the first reported human cowpox infection in the U.S. occurred in 2011 when a laboratory worker at the University of Illinois was exposed due to laboratory contamination (Reardon, 2011).

2) Smallpox Vaccines and Treatments

Historical studies suggest smallpox may have been identified as early as 10,000 B.C. in populated regions like Mesopotamia, and the disease continued its spread around the world with human movement (Shchelkunov, 2009). Eventually, cities with dense populations became breeding grounds for various viral and bacterial contagions. The first known treatment of smallpox was variolation. Voigt (2016) defines this as “the deliberate introduction of the infectious smallpox virus from the pustule of an infected person into a healthy, nonimmune person to induce a milder form of the disease.” Naturally, a complication of this treatment was that the person exposed to the virus perished from fully developed smallpox. Later in the 18th century, a doctor observed that infection with cowpox could provide resistance to smallpox, and a virus isolated from cows became the basis of early smallpox vaccines (Rusnock, 2016).

Today, the smallpox vaccine contains live VACV, which is the virus that was passed down through hundreds of years of growth in live animals, usually calves or horses. The vaccine is administered by depositing a droplet that contains the virus on the upper arm, followed by multiple pokes with a needle to induce a localized infection. People who are pregnant,

immunocompromised, or have any history of skin conditions like eczema are not eligible to receive the vaccine due to likely adverse reactions (Lederman et al., 2009). Serious complications can include an allergic response or spread of the infection to other areas on the body, while life threatening conditions manifest as progressive vaccinia, eczema vaccinatum, and postvaccinal encephalitis (Centers for Disease Control [CDC], 2017). The CDC (2017) compiled data using two studies from 1968 and found 1 in 1,000 people vaccinated experienced serious but not life-threatening reactions, and between 14 and 52 out of 1 million people vaccinated for the first time developed life-threatening reactions. In a more recent study with volunteers vaccinated as first responders, 1 in 450 people had to be hospitalized due to adverse reactions, and 1 death occurred per 13,000 vaccinations (Casey et al., 2005). For this reason, the vaccine is currently only available to laboratory workers, first responders, and military personnel, leaving the majority of the population unvaccinated and vulnerable to potential bioterrorism events. An alternative to standard smallpox vaccines containing VACV, such as ACAM2000, is a vaccine containing Modified Vaccinia Ankara (MVA) virus. This highly attenuated VACV strain was generated as a safer vaccine option: it is missing 15% of the parental genome and is replication deficient in human cells (Sánchez-Sampedro, 2015). However, high doses and multiple doses of MVA must be administered to elicit immune protection (Sánchez-Sampedro, 2015), and MVA does not induce the same type of immune response as VACV. Multiple epitopes that induce immunity in VACV vaccination are missing in MVA, so its protective efficacy against smallpox is unknown (Pugh, Keasey, Korman, Pittman, & Ulrich, 2014). Indeed its protective efficacy has been shown to be inferior to VACV (Earl et al., 2004; Hatch et al., 2013). Smallpox vaccines with improved efficacy and safety are needed.

Poxvirus drugs are the only option to treat infection, and they work by targeting specific stages in the viral life cycle. Cidofovir (CDV) is licensed for clinical use against cytomegalovirus, but it has been shown to be effective against other DNA viruses including poxviruses by inhibiting viral DNA synthesis (De Clercq, 2002). CDV has also been used clinically to treat Molluscum contagiosum virus infections in HIV patients and to combat extreme cases of eczema vaccinatum (Andrei & Snoeck, 2010). Side effects of treatment with CDV include kidney damage and eye inflammation (De Clercq, 2002). An additional concern is the use of CDV resistant mutant poxviruses, which can easily be made in a laboratory, as bioterrorism weapons. In a study, CDV resistant forms of camelpox, cowpox, monkeypox, and VACV were developed by replicating the viruses for at least 20 passages in cells in the presence of increasing concentrations of the drug (Smee, Sidwell, Kefauver, Bray, & Huggins, 2002).

The most promising poxvirus drug available is ST-246, also known as tecovirimat or Tpoxx, which was approved by the U.S. Food and Drug Administration (FDA) in 2018 and is the first and only FDA approved drug to treat smallpox (McNeil, 2018). Tpoxx prevents viral envelopment and cell to cell spread by targeting the poxvirus F13L/p37, a protein involved in the formation of enveloped virions (Grosenbach, Jordan, & Hruby, 2011; Roper & Moss, 1999). According to the FDA (2018), it is taken in capsule form twice daily for 14 days with the most frequent adverse reactions being headache and nausea. Despite its approval, Tpoxx has only been tested in animal models using non-variola Orthopoxviruses, and its effectiveness for treatment of smallpox in humans is unknown. Also, it has been shown that a single point mutation in the viral genome is sufficient to induce ST-246 resistance (Yakimovich, 2017). Although Tpoxx is a promising remedy for smallpox, the smallpox vaccines are the only proven method for preventing the disease.

3) Medical Applications of Poxviruses

Despite the pathogenic propensity of poxviruses, attenuated poxviruses can be utilized in a productive capacity for human health as preventative vaccines for infectious diseases and as therapeutic vectors (Gómez, Nájera, Krupa, & Esteban, 2008). Poxvirus vectors are beneficial because they create a robust immune response, can support large insertions of DNA, and they infect a variety of cell types (Tscharke et al., 2005). Additional advantages include lack of genomic integration, low cost and ease to manufacture and administer, and low prevalence of anti-vector immunity (García-Arriaza & Esteban, 2014). Currently, there are pox-based vaccines in development for a wide assortment of diseases including HIV, Malaria, Ebola, and Rabies. In 2009, a Phase III clinical trial was completed in Thailand for a canarypox based vaccine to prevent HIV-1, which showed 31.2% efficacy against HIV infection (Vaccari, Poonam, & Franchini, 2010). This is the only HIV vaccine that has ever shown efficacy in humans. More recently, a Phase 1/2 trial was performed in South Africa using a variation of the same HIV vaccine, and the results qualified the vaccine for Phase 2b/3 efficacy testing that began in 2018 in South Africa (Bekker et al., 2018).

Poxviruses can be effective in treating diseases such as cancer as well. The process of using a virus to selectively infect and lyse tumor cells is called oncolytic virotherapy, and it is becoming an increasingly popular anti-cancer therapy (Rahal & Musher, 2017; Yaghchi, Zhang, Alusi, Lemoine, & Wang, 2015). VACV is an ideal candidate because unlike other oncolytic viruses, it does not have a specific surface receptor for entry, allowing it to infect many different cell types (Yaghchi et al., 2015). In addition, cancer tends to create hypoxic environments, and studies have shown that VACV can still replicate and produce proteins effectively under hypoxic conditions (Yaghchi et al., 2015). There are also poxvirus based vaccines that promote an

immune response towards antigens upregulated during cancer development (Gómez et al., 2008; Zervos, Agle, Freistaedter, Jones, & Roper, 2016). Several poxvirus based cancer vaccines in clinical trials include TroVax for metastatic renal cancer, PROSTVAC for prostate cancer and PANVAC for patients with metastatic breast cancer (Guo et al., 2019).

4) Viral Virulence

Structurally, poxviruses are complex with a double-stranded, 130-400 kb DNA genome, which is packaged along with associated enzymes in large brick-shaped particles (Baxby, 1996; Roper, Payne, & Moss, 1996; Wolffe, Katz, Weisburg, & Moss, 1997). Of the genes that have been identified, it is estimated that ~30% are immunoregulatory (Bidgood & Mercer, 2015). This arsenal of proteins helps poxviruses repress the host immune response to promote viral replication and spread within the body and to other hosts. They target and inhibit various cellular processes such as apoptosis, cytokine and chemokine production, and T lymphocyte activity. Some VACV proteins are secreted and bind chemokines or cytokines extracellularly such as VACV B8 and B18, which bind interferon- gamma (IFN- γ) or type I interferons (IFNs) (Albarnez, Torres, & Smith, 2018). IFNs are important for the induction of IFN-stimulated genes to produce an anti-viral response. Other proteins work intracellularly to inhibit innate signaling pathways that stimulate the production of IFNs and cytokines or activate programmed cell death (Albarnez et al., 2018). VACV protein C6 has been found to inhibit the activation of IRF3 and IRF7 by binding adaptor proteins needed to initiate upstream kinases, preventing the production of type I IFNs (Albarnez et al., 2018). Despite the progress that has been made, further research is needed to study virulence genes and their mechanisms.

5) A35 and O1L Poxvirus Genes

Our laboratory identified several poxvirus genes using bioinformatics that were hypothesized to be immunoregulatory genes including A35 and O1L. These genes are highly conserved in mammalian tropic poxviruses, suggesting that they have an important conserved function. They are not conserved in poxviruses infecting lower vertebrates, which do not have well developed adaptive immune systems. In addition, these genes have not been identified in screens detecting genes required for replication, suggesting that they are not required for replication but rather for accessory functions. Homologs of A35 and O1L are also not found in other viruses including herpesviruses. Herpesviruses are believed to have evolved separately from poxviruses before the development of the specific mammalian immune system, meaning poxviruses would not share genes that regulate the adaptive immune response with Herpesviruses. Together this evidence suggests VACV A35 and O1L genes may be immunoregulatory, and this hypothesis was tested.

Characterization of A35

The A35 gene, also known as gene 158 in VACV-Western Reserve (WR) Strain, was previously characterized by our laboratory to express a 23 kDa non-glycosylated, hydrophobic protein early in infection that is not secreted, suggesting an intracellular function (Roper, 2006). The protein was detected in wild type WR virus infected cells for at least 18 h post infection (hpi) (Roper, 2006). While the hydrophobic nature of A35 initially implied it could be located in a membrane, studies showed it is likely a soluble cytoplasmic protein (Roper, 2006). To determine the function of A35, an A35 deletion mutant virus (A35Del) was generated by replacing the A35 gene with an *E.coli* gene as a selective marker (Roper, 2006). The A35Del virus was screened using polymerase chain reaction (PCR) for the presence of the *E. coli* gene and the absence of the A35 gene with primers flanking the A35 locus, and protein expression

was analyzed using Western blot to ensure A35 had successfully been deleted (Roper, 2006). The A35Del virus formed wild-type plaques and replicated normally in a number of cell lines and primary cells from various hosts, indicating A35 is not required for replication or spread *in vitro* (Rehm, Connor, Jones, Yimbu, & Roper, 2010; Roper, 2006). While A35 was not required for replication *in vitro*, it may have still been necessary for replication *in vivo*. Therefore, mice were infected intranasally with WR or A35Del, and nose, lungs, brain and spleen were harvested on days 1, 2, and 3 post challenge to look at early replication *in vivo* (Rehm, Jones, Tripp, Metcalf, & Roper, 2010). Organ titers were similar between the WR and A35Del viruses at this time point, which suggests A35 is also unnecessary for early replication and spread *in vivo* (Rehm, Jones et al., 2010). To better understand how A35 affects the host, the Roper laboratory performed virulence studies by challenging mice intranasally with WR or A35Del and monitoring weight loss. At a dose of 10^4 plaque forming units (pfu)/mouse, A35Del infected mice maintained their weight, while WR infected mice experienced significant weight loss (20-30%), and two WR infected mice reached 30% weight loss and were sacrificed (Roper, 2006). The attenuation of A35Del was seen at higher challenge doses as well, confirming A35 is required for full virulence in mice via the intranasal route; and in turn it requires 1000x more A35Del virus to reach wild-type virulence (Roper, 2006). The Roper laboratory also studied the role of A35 in intraperitoneal infection and found A35 is required for full virulence via this infection route as well (Rehm, Jones et al., 2010). The lack of any phenotype in tissue culture and the importance of A35 in virulence in mice suggested it may be involved in regulating the immune system, and this hypothesis was tested. Since A35 is conserved in mammalian tropic poxviruses and not in poxviruses infecting lower vertebrates, the effects of A35 on the adaptive immune response were explored.

For VACV infection, CD4⁺ T cell and major histocompatibility complex (MHC) class II expression have been shown to be crucial for protection against poxviruses (Wyatt, Earl, Eller, & Moss, 2004). The Roper laboratory and others have shown that VACV inhibits MHC class II antigen presentation by peritoneal exudate cells (PEC), a B lymphocyte line, splenocytes, and dendritic cells to CD4⁺ T lymphocytes (Rehm et al., 2009), similar to the work of others using a transformed human B cell line (Li et al., 2005). To investigate if A35 affects antigen presentation, experiments were performed using an *in vitro* MHC class II restricted model with infected rat PEC presenting antigen to RSL.11 T cells (Rehm, Connor et al., 2010). Supernatants were collected and tested for interleukin 2 (IL-2) and nitric oxide (NO) as indicators of antigen presentation (Rehm, Connor et al., 2010). Data showed A35 reduces IL-2 and NO production as well as decreases a variety of other subsequent cytokines released due to antigen presentation (Rehm, Connor et al., 2010). The Roper laboratory investigated this inhibition and found that A35 does not directly affect the T lymphocytes but exerts its influence on the antigen presenting cell (APC) (Rehm, Connor et al., 2010). They looked at the ability of A35Del to infect and replicate in PEC, as well as the ability to promote cell death and apoptosis as possible explanations for the observed differences in antigen presentation; however the results were similar to WR (Rehm, Connor et al., 2010). Additionally, when PEC were infected and stimulated to produce NO via alternate pathways, A35 had no effect on the NO response. The fact that A35 only reduced NO as a result of antigen presentation and not other stimuli suggested that A35 inhibits antigen presentation (Rehm, Connor et al., 2010). Dr. Roper hypothesized that if A35 blocks antigen presentation to CD4⁺ T cells, it would also decrease subsequent immune responses dependent on T helper lymphocytes. *In vivo* experiments showed that A35 inhibits anti-viral antibody production, IFN- γ secreting T cells, cytotoxic T lymphocyte response, and

proliferation of lymphocytes in the spleen, which could all be subsequent effects related to the inhibition of MHC class II antigen presentation (Rehm, Jones et al., 2010; Rehm & Roper, 2011)

Current A35 studies are focused on determining the mechanism of A35 in antigen presentation. Previous data by the Roper laboratory revealed A35 decreases the amount of peptide presented in the cleft of MHC class II, resulting in an increase in class II associated invariant chain peptide (CLIP) detected on the surface of cells (Rehm, Connor et al., 2010). When considered with immunofluorescence data that showed A35 localizes to APC endosomes, it is possible A35 may interact or interfere with proteins in endosomes important for antigen presentation (Rehm, Connor et al., 2010). While our laboratory has shown A35 dampens antigen presentation-induced responses, data also showed that A35 was not the only VACV gene acting on the adaptive immune response (Rehm, Connor et al., 2010). The Roper laboratory identified additional potential immunoregulatory genes that are being characterized as well.

Characterization of OIL

OIL is another poxvirus gene that appears to have an important role in the VACV life cycle. It encodes a large protein (~78 kDa, 666 amino acids) which is transcribed early and sustained late during infection (Schweneker et al., 2012), but the function is currently unknown. Protein motif prediction programs predict that OIL contains one transmembrane region from approximately AA 570 to 596, suggesting that it may be membrane associated. It also contains a leucine zipper motif and potential bipartite nuclear localization signal (Schweneker et al., 2012). OIL was detected in the cytoplasmic and nuclear fractions (Roper lab unpublished); however, it is unknown whether OIL enters the nucleus or is binding the outside of the nucleus or poxvirus DNA. The Roper lab performed immunofluorescence microscopy to examine the intracellular localization of OIL and saw diffuse staining in the cytoplasm, which does not

provide support for a nuclear function of O1L. As with the VACV A35 gene, the Roper laboratory created an O1L deletion mutant virus (O1LDel) to investigate the role of O1L in poxvirus infection. To do this, the O1L gene was removed from VACV and replaced with the gene for enhanced green fluorescent protein (egfp) as a screening marker. The presence of egfp and the absence of O1L at the O1L locus were confirmed using PCR in 2 independent O1LDel mutants, and egfp expression in O1LDel plaques was examined using a fluorescence microscope. Western blots were also performed to confirm the expression of the O1L protein in WR and its absence in the O1LDel mutants. The ability of the O1LDel virus to replicate and spread was tested in African green monkey kidney cells (BS-C-1) as well as several other cell lines and found to be similar to wild type. While the plaque diameters for both viruses were comparable, it was noted that in some cases, the O1LDel plaques appeared to have less cell clearance in the center. One step and multi-step growth curves performed with WR and O1LDel infected cells also revealed no difference in the amount of cell associated or free infectious virus produced, which indicated O1L does not appear to affect replication or spread *in vitro*.

To look at *in vivo* effects of O1L, the Roper laboratory infected mice intranasally with O1LDel or WR viruses and measured weight loss over time. O1LDel infected mice lost weight within the first week of infection but recovered, while WR infected mice experienced significant weight loss and died, revealing O1L is required for full virulence in mice. These results have been confirmed by another group using mice infected with a different strain of VACV, Chorioallantois VACV Ankara virus (CVA), and an O1L deletion mutant virus (CVA-O1LDel) (Schwenker et al., 2012). Since O1L appears to be important for VACV virulence, the Roper laboratory investigated the effects of O1L on replication, spread, and tropism *in vivo* by infecting mice for 7 days and performing organ titers. Titers between WR and two O1LDel mutant

viruses were similar in hearts, spleens, brains, and noses of mice, but significantly lower in both O1LDel infected lungs in one experiment, suggesting O1L may affect the ability of the virus to replicate in some tissues. Another lab found there was no significant difference in viral titer between CVA and CVA-O1LDel in the lungs, but they did find a significant difference in the number of infectious virus particles in the ovaries at day 6 (Schweneker et al., 2012). While some small effects of O1L on replication and spread have been reported, we do not know if it is enough to explain its function and conservations.

Schweneker et al. (2012) found that O1L causes sustained activation of the Raf/MEK/ERK pathway, which is important in cell death and survival, proliferation, differentiation, migration, and motility (Shaul & Seger, 2007; Vial & Pouyssegur, 2004; Yoon & Seger, 2006). In cells infected with CVA-O1LDel, there was little to no phosphorylated ERK detected, compared to CVA infected cells which showed sustained ERK activation (Schweneker et al., 2012). There are several RNA and DNA viruses that activate this pathway during infection to promote replication (Schweneker et al., 2012). The Schweneker laboratory (2012) examined the replication and spread of CVA-O1LDel *in vitro* and noted a difference in plaque morphology, seen previously by the Roper laboratory. They hypothesized that the reduction in plaque size observed in CVA-O1LDel plaques was related to reduced replicative capacity or impaired cell-cell spread, based on data showing a small significant difference in viral yield between CVA-O1LDel and CVA at only 2 middle points out of 6 time points (Schweneker et al., 2012). In the same experiment, O1LDel infected cells had increased viral titer compared to wild type at a later time point. While the differences in viral yield were significant, other laboratories studying VACV genes necessary for replication have seen anywhere from 2 to 3 log differences in viral yield (Gammon et al., 2010; Liu, Katsafanas, Liu, Wyatt, & Moss, 2015). Also, the

Roper laboratory has performed one step and multi-step growth curves in a variety of cell lines and seen no effects of O1L on replication or spread. Therefore, my work has been focused on further characterizing the effects of O1L on the virus life cycle including effects on cell death and survival, proliferation, differentiation, migration, and motility.

Both A35 and O1L remain genes of interest for the Roper laboratory. To further assess their functions in the VACV life cycle, antigen presentation experiments were performed utilizing a new experimental model. In the place of rat PEC, splenocytes, dendritic cells or human B cell lines, mouse bone marrow dendritic cells (BMDC) were used as APC because they are important natural APC and are extremely efficient at presenting antigen and priming T cell responses. The APC were infected with WR, A35Del, or O1LDel viruses to present viral antigen to virus specific CD4⁺ T cells that were amplified *in vivo*. Using antigen specific T cells allowed us to see if the genes affect the T lymphocyte response in the mouse as well as to test if A35 or O1L affect recall antigen presentation *in vitro*. Because BMDC were utilized as APC, metabolism assays were performed to look at VACV cell killing in BMDC, along with one-step growth curves to determine the ability of VACV to replicate in this cell type. O1L effects on anti-viral antibody production, integrin expression, plaque morphology, cell killing, antigen presentation- induced cytokine production, and innate immunity were examined to further elucidate its function.

CHAPTER 2: MATERIALS AND METHODS

Cells and Virus

BS-C-1 cells were maintained in minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS), 2 mM Glutamax, and 100 Units/mL of Penicillin and Streptomycin antibiotic (referred to as complete MEM [cMEM]). Cytotoxic T lymphocyte line cells (CTLL) were retrieved from liquid nitrogen storage and maintained in Roswell Park Memorial Institute media (RPMI) supplemented with the additives listed above, along with 2-betamercaptoethanol to make complete RPMI (cRPMI) and 0.5% IL-2. For bone marrow dendritic cells (BMDC), bone marrow was harvested from mouse femurs. The bones were cleaned and placed in a dish with 5 mL of cold cRPMI. After removing the ends, a 1 mL syringe was used to extract the bone marrow cells, and they were plated in cRPMI and 1-2% granulocyte macrophage colony stimulating factor (GM-CSF). On Days 3 and 6, additional media with GM-CSF were added to feed the BMDC. On Days 6-8, the BMDC were collected for use in experiments. All cell types were incubated at 37°C with 5% CO₂. VACV viruses used including WR, A35Del, and O1LDel were from stocks previously prepared by the Roper laboratory (Roper, 2006) and stored at -80°C. The Roper lab prepares large stocks of virus to use for years in order to avoid any possible stock variations. Titers are determined periodically and remain stable for years. The A35Del and O1LDel viruses utilized in these experiments were previously analyzed using PCR and Western blot to ensure that the gene in question had been successfully deleted. In addition, their phenotype was checked during these experiments.

One Step Growth Curve

In a 15 mL conical tube, BMDC were infected with sonicated crude WR or A35Del at a multiplicity of infection (MOI) of 10 plaque forming units (pfu)/cell and a final volume of 1 mL. The tubes were incubated at 37°C 5% CO₂ for 1.5 h, tilted at an angle with loose caps. Following incubation, the tubes were spun at 320 x g for 5 min, and the BMDC were resuspended in warm cRPMI + 0.6% GMCSF. In a 96 well plate, 150 ul of cells were plated in duplicate per group for a total of 1×10^5 cells/well. Individual wells were harvested by scraping the bottom in zigzag motions with the pipette tip at specified time points. Afterwards, the media were pipetted into a labeled Eppendorf tube and spun at 320 x g for 1 min. 50 ul of supernatant were transferred to a separate labeled Eppendorf tube, and both the supernatant and cell lysate tubes were frozen at -20°C for viral plaque assays.

Viral Plaque Assay

Confluent BS-C-1 monolayers were prepared in 6 well plates. On the day of infection, the media in the wells were aspirated and replaced with 1 mL of fresh cMEM. Samples were freeze/thawed a total of 3 times and sonicated for 30 pulses. Then, cell lysate or supernatant samples were added to the monolayers per the infection plan to produce countable wells. Plates were placed in an incubator at 37°C 5% CO₂ and swirled several times during the first hour of infection to ensure virus attachment. After 40-48 h of infection, the wells were stained with 1 mL of 0.1% crystal violet in 20% ethanol for 5 min. The crystal violet was aspirated, and the plates were set out to dry at room temperature (RT). Plaques in each well were counted at least twice, and the average number of plaques was calculated. This number was divided by the amount of sample added to the BS-C-1 cells to determine the titer in pfu/mL. Titers were

adjusted by the actual volume present in the 96 well plate from the one step growth curve to give pfu/well.

Antigen Presentation

Groups of C57BL/6 mice (n= 5) were infected intranasally with purified WR, A35Del, or O1LDel for 1, 2, 3, or 4 weeks to allow CD4⁺ T lymphocytes to activate, proliferate, and differentiate in order to amplify VACV specific T cells. Later time points provided time for the T cells to stabilize from the first activation. Infected BMDC were used as antigen presenting cells (APC). On the day of the antigen presentation assay, BMDC were harvested and either uninfected (UN) or infected with purified WR, A35Del, or O1LDel in 15 mL conical tubes at an MOI = 1. The tubes were incubated at 37°C 5% CO₂ for 4-5 h. Following incubation, BMDC were plated in 96 well plates at 10,000-100,000 cells/well.

Mouse spleens were harvested from infected mice and placed in 50 mL conical tubes with cold cRPMI. Spleens were then macerated in a culture dish using a 3 mL syringe and transferred to a 50 mL conical tube. The culture dish was washed 1-2 times with 5 mL of cRPMI to retrieve as many cells as possible. After allowing the chunks of tissue to settle, the supernatants were collected and centrifuged at 350 x g for 5-8 min at 4°C. Cells were resuspended in 5 mL of ACK Buffer (1.66% NH₄Cl in deionized water, pH 7.2) to lyse red blood cells and incubated on ice for 5 min. 10 mL of cRPMI was added to stop the lysis, and the tubes were spun at 350 x g for 5-8 min, then the cells were resuspended in 1 mL isolation buffer. Dynabeads Untouched Mouse CD4 Cells kit (Thermo Scientific, 11415D) was used to isolate the CD4⁺ T cells. Cells were processed per the manufacturer's instructions. Following isolation, the T cells were resuspended in cold cRPMI and counted. An equivalent number of T cells was added to each well in a volume of 100 ul, and the plates were incubated at 37°C 5% CO₂. 24-36

h and 48-55 h post addition of T cells, 50 ul of supernatants were harvested using a multichannel pipette and frozen in a humidified bag to be used in cytokine assays.

Antigen Presentation with Splenocytes only

Mice (n= 5) were infected with WR or O1LDel as described previously. The spleens were then harvested and processed to create a single cell suspension. In a 96 well plate, 2×10^6 splenocytes from WR or O1LDel infected mice were added to each well and either UN or infected with WR or O1LDel at an MOI of 0.5 pfu/cell. The cells were then incubated at 37°C 5% CO₂, and 50 ul of supernatants were harvested at approximately 30 and 46 h post infection (hpi) to be measured for cytokine production.

Antigen Presentation to Transgenic T Cells

Splenocytes or T cells from 2D2 transgenic mice and antigen were donated by Kayla DeOca and Cody Moorman from the Mannie laboratory for use in antigen presentation assays. Approximately 2×10^5 splenocytes were added per well and either UN or infected with WR or O1LDel at a MOI = 0.5 for ~3 h. Then, the cells were pulsed with 320 nM of neurofilament medium polypeptide (NFM13-37), and supernatant samples were taken at ~43 h. In another experimental design, 100,000 BMDC were used as APC and infected with WR or O1LDel at an MOI = 1 for 4 h, then pulsed with 5 uM myelin oligodendrocyte glycoprotein (MOG35-55) or 500 nM NFM13-37. After incubating with antigen for 30 min, 4×10^4 2D2 T cells were added, and supernatants were harvested 24 h and ~46 h later to be tested for cytokine production.

Metabolism (MTS) assay

The plate reader was turned on and set up approximately 10 min before use. 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS, Promega) was retrieved from -20°C storage, wrapped in foil due to light sensitivity and

thawed in a warm water bath. Then, 10 ul of MTS were added to each well via a multichannel pipette or combi pipette in a dark hood to quantify cellular metabolism. Absorbances were measured at 492 nm until numbers plateaued. Plates were kept at 37°C 5% CO₂, protected from light in between readings.

CTLL IL-2 Bioassay

CTLL were expanded in cRPMI + 0.5% IL-2 in a T-25 flask until the cells were no longer proliferating, at least 60% dead. To remove any remaining IL-2, the cells were transferred to a 50 mL conical tube and washed with an equivalent volume of cRPMI (no IL-2). CTLL were spun at 500 x g for 8 min, resuspended in cRPMI (no IL-2), and then counted 1:10 using trypan blue. 96 well plates containing 50 ul/well of supernatant were retrieved from -20°C storage and brought to RT. 3 wells with 50 ul cRPMI (no IL-2) and 3 wells with 50 ul cRPMI + 0.5% IL-2 were added to each plate to serve as negative and positive controls, respectively. Using a multichannel pipette, approximately 5×10^4 CTLL were added in 150 ul to the samples and controls. The wells were examined under a microscope to ensure each well had an equivalent amount of cells and then incubated at 37°C 5% CO₂. The plates were inspected daily to observe any differences in cell number or morphology within sample and control groups. When differences were evident, an MTS assay was performed to measure proliferation in response to IL-2 in the supernatants.

Nitric Oxide (NO) Assay

96 well plates containing 50 ul of supernatant/well were retrieved from -20°C and thawed at RT. Approximately 30 min prior to use, the sulfanilamide and NED solutions were taken out of 4°C storage and brought to RT as well. For a positive control, the nitrite standard included in the Greiss Reagent System kit (Promega, G2930) was diluted in cRPMI, and 50 ul were pipetted into

a well on each plate, along with 50 ul of cRPMI to be used as a negative control. The Greiss reagent was prepared by mixing the sulfanilamide and NED solutions in a 50 mL conical tube to total 25 ul of each solution per well. This step was completed in a dark hood as the reagents are light sensitive. Using a multichannel pipette, 50 ul of the Greiss mixture were added to all sample and control wells. Absorbances were measured at 550 nm using a plate reader. Color change was monitored, and the plates were read as needed to capture differences between groups.

Anti-viral Antibody ELISA

Groups of C57BL/6 mice (n=5) were infected intranasally with purified WR, A35Del, or O1LDe1. An additional 2 mice were mock infected with phosphate buffered saline (PBS) as a control. Blood was collected 1, 2, 3, or 4 weeks post infection via a heart stick, and sera were isolated. To measure anti-viral antibody, Immulon 96 well flat-bottom enzyme-linked immunosorbent assay (ELISA) plates (Thermo Scientific) were coated with 0.1 ul/well sonicated crude WR and 49.9 ul/well ELISA Coating Buffer (1L deionized water w/ 10.3 g H₂B04, 7.31 g NaCl, pH 8.5). The plates were covered with parafilm and placed in a humidified bag to incubate at 4°C for up to one week. On the day of testing, 100 ul of ELISA Blocking Buffer (1xPBS w/ 2% FBS, 0.1% NaN₃) were added to each well and incubated at RT for 30 min in a humidified bag. The sera samples were retrieved from -20°C storage, thawed using a warm water bath, and kept on ice. At the end of the incubation, 2 washes were performed using ELISA Wash Buffer (1xPBS w/ 0.02% Tween20, 0.1% NaN₃) with a 1 min incubation in between. Sterile PBS was pipetted per the plate design to keep the wells hydrated, and 10 ul of sera were added to 140 ul of PBS in the first row for a total well volume of 150 ul. The sera were then diluted 1:3 down the plate by transferring 50 ul between wells. A well with 1 ul of known

positive sera was used as a positive control, while the sera from PBS infected mice acted as a negative control. The plates were then covered in parafilm and incubated at RT for 2 h in a humidified bag.

The secondary antibody solution was prepared using goat anti-mouse Ig(H+L) conjugated to alkaline phosphatase (Southern Biotech, 1010-04, detects mouse IgM, IgG, and IgA), goat anti-mouse IgG conjugated to alkaline phosphatase (Southern Biotech, 1030-04), and ELISA Blocking Buffer. After washing, 0.05 ul of each antibody in ELISA Blocking Buffer were added per well with a multichannel pipette for a total volume of 50 ul/well. The plates were covered with parafilm and incubated at RT for 1.5 h in a humidified bag. During the third incubation, the substrate solution was prepared using a Bio-Rad Alkaline Phosphate kit. The substrate was tested for reactivity by combining a drop of substrate with secondary antibody to observe color change. Following a third wash, 50 ul of substrate were added to each well. The plates were then read at 405 and 690 nm every 5-10 min until absorbances plateaued. Mice with antibody similar to the PBS control were excluded based on the following rule: if at dilution 2 (2.2 ul serum/well), the amount of antibody detected was less than 1.5x the average PBS value at the same point, the mouse was considered UN and was omitted.

Flow Cytometry

BMDC were harvested into 4 tubes labeled UN, WR, A35Del, O1LDe1, or unstained/secondary only. Cells were infected at an MOI = 4 and incubated overnight (O/N), tilted at an angle with loose caps. The next day, 1 mL of cold PAB (PBS, 1% FBS, 0.1% NaN₃) was added to each tube, flicked to mix, and spun at 320 x g for 8 min at 4°C. The cells were resuspended in PAB, and 100 ul of cells were added to labeled Eppendorf tubes. BMDC were stained with one of the following primary antibodies in 200 ul: rat anti- mouse CD11a (α L) (Invitrogen, 14-0111-82),

rat anti-mouse CD18 (β 2) (Invitrogen, 14-0181-82), or rat anti- mouse CD29 (β 1) (Invitrogen, 14-0292-82). For CD11a and CD18, 3 μ l of antibody were added per tube, and 1 μ l of anti-CD29 was added per tube. The cells were incubated for 1 h on ice, and flicked twice to ensure binding. During the incubation, the secondary antibody, goat anti-rat (Heavy + Light) cross absorbed Alexa Fluor 594 (Invitrogen, A11007), was diluted 1:250 in PAB. Cells were washed with 1 mL of PAB and spun at 500 x g for 5 min at 4°C. BMDC were then resuspended in 200 μ l of the secondary antibody mixture and incubated for 1 h on ice. Two washes with PAB were completed as described above. Then, the cells were resuspended in 400 μ l of PAB and transferred to FACS tubes. Stained BMDC were run on the FACS Aria Fusion using the settings for PE-CF594-A, which matched the emission wavelength for Alexa 594. Data were analyzed via FlowJo software.

Cell Killing

CTLL, BMDC, and splenocytes were prepared as described previously. In a 96 well plate, cells were either UN or infected with WR or O1LDel at an MOI = 5 or 6. Cells were incubated for ~48 h or until differences were evident between groups, and an MTS assay was performed to measure cellular viability.

Cytokine Bead Assay

A Cytometric Bead Assay Mouse Th1/Th2/Th17 Cytokine Kit (BD, 560485) was used to measure IL-2, interleukin 4 (IL-4), interleukin 6 (IL-6), interferon gamma (IFN- γ), tumor necrosis factor (TNF), interleukin 17A (IL-17A), and interleukin 10 (IL-10) in antigen presentation supernatants. Briefly, supernatant samples were retrieved from -20°C storage and brought to RT. Beads with unique mean fluorescent intensities and coated with antibody for

each cytokine were combined in a 15 mL tube. 50 ul of the bead mixture were added to 50 ul of sample, followed by 50 ul of the phycoerythrin detection reagent. The samples were incubated for 2 h at RT in a dark hood. After washing, the samples were resuspended in 300 ul of wash buffer and transferred to FACS tubes. Cytokine production was measured using Flow cytometry, and the data were analyzed via FlowJo.

Worm Hemolymph Assay

Waxworms were provided by the Pesci laboratory and kept in a 50 mL conical tube with air holes. BS-C-1 cells were added to a 96 well plate and allowed to adhere for approximately 4 h. Hemolymph was harvested by amputating the heads of the worms with scissors and allowing the liquid to drain in to a culture dish. The hemolymph was then transferred to an Eppendorf tube. Approximately 700 ul of hemolymph was collected from 20-25 worms. Several conditions with virus and hemolymph were tested: 1) 5.5-6 ul of virus (WR or O1LDel) were incubated with varying amounts of hemolymph (1, 5, 10, or 15 ul) for 10 min and then added to the cells, or 2) cells were pre-incubated with 5 ul of hemolymph for 10 min followed by infection with 5.5-6 ul of virus. In each case, the calculated amount of virus added was 100 pfu/well. The plates were incubated at 37°C for ~21 h, then put in a humidified bag, wrapped in parafilm, and frozen at -20°C. The samples were freeze/thawed a total of 3 times to create a cell lysate. During the second thaw, the samples were transferred to labeled Eppendorf tubes to facilitate testing. To titer, the Eppendorf tubes were sonicated for 30 pulses, and viral plaque assays were performed to measure the number of infectious virus particles/well.

Statistical Analysis

Data were analyzed using Microsoft Excel via a one-way or two-way ANOVA as appropriate and unpaired two-tailed Student's T-Tests. A p value less than 0.05 ($p < 0.05$) was considered statistically significant.

CHAPTER 3: RESULTS

VACV A35 Gene

VACV Replication in BMDC

Our lab has shown that the VACV A35 gene inhibits antigen presentation by rat macrophages, and we wanted to determine its effects on BMDC, mouse primary antigen presenting cells (APC) believed to be key professional APC *in vivo*. We first looked at effects on BMDC killing. BMDC were infected with the wild type WR virus or A35Del virus, and MTS was added at ~48 h post infection (hpi) to measure cellular metabolism. BMDC infected with WR or A35Del had similarly reduced metabolism compared to UN cells (Figure 1). We did not see any evidence that A35 increased cell killing. As previously shown for PEC (Rehm, Connor et al., 2010), these data suggest that A35 does not block antigen presentation by increasing killing in the APC.

Next, we determined whether VACV is able to replicate in BMDC and to evaluate if A35 affects viral replication in this cell type. In 4 experiments, one step growth curves were completed by infecting BMDC with WR or A35Del at an MOI of 10 and harvesting samples over time to measure the number of infectious virus particles present in the cell lysate and supernatants. A high MOI was used to ensure the majority of cells became infected, so that we could examine the number of plaque forming units (pfu) resulting from one cycle of viral replication. One step growth curves were completed using BMDC from two different mouse strains: BALB/c and C57BL/6. In both mouse strains, the number of infectious virus particles produced by WR and A35Del infected cells were similar to each other in the cell lysate and supernatant samples (Figure 2). The difference seen at 24 hpi in BALB/c BMDC was not reproducible. Cell associated virus and free infectious virus stayed relatively constant over time for both WR and A35Del, and pfu/well remained significantly below (less than 1/10th) the

amount of input virus. This indicates that there was no virus replication in BMDC under these conditions and no effect of A35 on replication. Normally, VACV replication begins shortly after infection, and the release of infectious progeny virus begins approximately 8 hpi (Roper, 2006). In past studies with BS-C-1 cells, which are permissive to VACV infection, the Roper laboratory has seen as much as a 100-fold increase in titer by 30 hpi (Rehm, Connor et al., 2010). Typically, permissive cells give approximately 100-fold increase in viral titers (Figure 25), so there is no indication that VACV WR can replicate in these BMDC (C57BL/6 or BALB/c mice).

Effects of A35 on Antigen Presentation and T Cell Response

To further test the hypothesis that A35 inhibits antigen presentation and subsequent T cell response, we infected mice intranasally with WR or A35Del viruses to induce an immune response and amplify virus specific CD4⁺ T lymphocytes. In this way, we could test the ability of these 2 viruses to induce T cell activation and proliferation *in vivo*. Next 1, 2, 3, or 4 weeks later, CD4⁺ T lymphocytes were isolated and each re-stimulated with BMDC infected with WR or A35Del. This would test both the *in vivo* stimulation of T lymphocytes and the ability of BMDC infected with virus (with or without the A35 gene present) to re-stimulate the T cells *ex vivo*. After approximately 24-48 h, supernatants were collected to evaluate cytokine production including interleukin 2 (IL-2) and nitric oxide (NO) as a measure of antigen presentation. Compared to the unstimulated control (BMDC with no virus antigen to re-stimulate the T lymphocytes), CD4⁺ T cells from infected mice were re-stimulated to release IL-2 by infected BMDC. Mice infected with A35Del virus had a significantly better ($p < 0.05$) T cell response compared to wild type WR infected mice at both 24 h and 48 h after *in vitro* stimulation (Figure 3). However, there was no difference in IL-2 production between T cells re-stimulated with BMDC infected with WR versus A35Del. Later reads for the 24 h supernatants were examined

and the absorbance values did not exceed 1 OD, suggesting that the MTS reaction had plateaued at the time point shown in Figure 3A. By 48 h post addition of T cells, there was an increase in IL-2 compared to 24 h, and absorbance values did exceed 1 OD. Overall, these results suggest that A35 inhibits the T cell response *in vivo*, but does not appear to affect T cell responses in recall antigen presentation *in vitro* for this model.

Supernatants were also tested for NO, which is released by some APC and can be used as another indicator of antigen presentation (Rehm et al., 2009; Rehm, Connor et al., 2010). The positive nitrite control reacted as expected and produced a pink color almost instantly after the Greiss mixture was added, indicating the Greiss reaction was proceeding properly; but no NO was detected in the supernatants from the antigen presentation experiments above background media and Greiss reagent (Figure 4). To test if there was some interfering factor in the supernatants, a small amount of positive control NO was added to one of the sample wells. The well turned color within seconds, revealing the reaction was not inhibited by anything in the supernatant. NO production has been previously induced in antigen presentation experiments in our laboratory using rat PEC (Rehm et al., 2009; Rehm, Connor et al., 2010), but we did not detect it in this BMDC model, even though we used BMDC that were differentiated for 6-8 days. While no NO was detected, IL-2 was present in the supernatants, which supports that antigen presentation occurred. The IL-2 data collected provides evidence that A35 in VACV inhibits the activation, amplification, and/or differentiation of the virus specific T lymphocyte response *in vivo*.

A35 and Anti-viral Antibody Production

Since A35 inhibited T cell response, the effect of VACV A35 on anti-viral antibody production was also measured. To do this, blood was harvested from mice infected with WR, A35Del, or

mock infected with phosphate buffered saline (PBS) for the antigen presentation experiments at 1, 2, 3, and 4 weeks after infection. Sera were collected, and anti-viral antibody production was measured via ELISA. Mice infected with A35Del virus had increased antibody production compared to PBS or WR infected mice in weeks 1-4 (Figure 5). Sera from mice infected for 3 weeks showed the most significant difference ($p < 0.01$) in anti-viral antibody between WR and A35Del infected mice. For sera from WR infected mice, there was an increase in antibody between week 1 and week 2, at which point antibody production remained similar for the subsequent weeks. At each time point, anti-viral antibody induced by infection of mice with WR was lower than sera from A35Del infected mice, while PBS treated mice remained constant at an absorbance of 0-0.2 OD and was used as a negative control. The data were further analyzed by finding the line of best fit for the dilution curves in week 3 and solving for the amount of sera from WR or A35Del infected mice necessary to reach an absorbance of 0.4 OD. The calculations revealed that it takes over 50 times more sera from WR infected mice compared to A35Del infected mice to reach the same absorbance value (Figure 6), confirming our previous data that A35Del infected mice produced significantly more anti-viral antibody (Rehm, Jones et al., 2010; Rehm & Roper, 2011). These data indicate that A35 in VACV inhibits anti-viral antibody production during an infection of mice.

Effect of A35 on Integrin Expression

While A35 plays a role in inhibiting the adaptive immune response, the mechanism remains unclear. A35 caused little to no decrease in MHC class II and B7.2 (CD86) expression on APC (Rehm, Connor et al., 2010), but integrin protein expression had not been previously tested. To determine how A35 is inhibiting T cell response and anti-viral antibody production, integrin expression on BMDC was examined by measuring expression of CD11a, CD18, and CD29

integrins. CD11a and CD18 heterodimerize to create the integrin complex lymphocyte function associated antigen 1 (LFA-1), which then binds intercellular adhesion molecule 1 (ICAM-1) during antigen presentation and brings the APC and T cell into close proximity. CD29 interacts with CD49D to create very late antigen 4 (VLA-4) that binds vascular cell adhesion molecule 1 (VCAM-1) on endothelial cells and helps leukocytes travel from the bloodstream into the tissue. To look at the effects of A35 on expression, BMDC were infected with WR or A35Del viruses and stained with a primary antibody for each integrin, followed by a secondary antibody to measure protein levels by flow cytometry. Compared to the control (BMDC stained with secondary antibody only), we were able to detect CD11a, CD18, and CD29 expression on BMDC, evidenced by the sample peaks (UN, WR, A35Del) shifting to the right (Figure 7). However, there was no difference in integrin expression between UN BMDC and WR or A35Del infected BMDC, providing no evidence that VACV infection or the A35 protein inhibits expression of the three integrins on this cell type. Expression of A35 in VACV infected BMDC was confirmed by Western blot (data not shown).

VACV O1L Gene

Role of O1L in Antigen Presentation and T Cell Response

Similar to A35, O1L is conserved in mammalian tropic poxviruses, unique to poxviruses, and there is no evidence that it is required for replication. The Roper laboratory showed that O1L is a virulence factor and hypothesized that O1L may be immunoregulatory as well. To investigate the effects of VACV O1L on antigen presentation and T lymphocyte response, antigen presentation assays were performed, as described above. Briefly, C57BL/6 male mice were infected with WR or O1LDel for 1, 2, 3, and 4 weeks, and CD4⁺ T cells were isolated from infected mice. The CD4⁺ T cells were then re-stimulated using 100,000 BMDC infected with

WR or O1LDel, and supernatant samples were collected to test for cytokine production. Compared to the unstimulated control (BMDC with no virus antigen to re-stimulate the T lymphocytes), CD4⁺ T cells from infected mice were re-stimulated to produce IL-2. Mice infected with O1LDel had similar T cell responses to wild type WR infected mice at 24 h, regardless of which virus was used to re-stimulate the T lymphocytes (Figure 8A); and there was no statistically significant difference in IL-2 production. While absorbance values are below 1 OD in the figure shown, later readings did not exceed 1, suggesting that the reaction had plateaued. For supernatants harvested at approximately 48 h (Figure 8B), BMDC infected with WR re-stimulated T cells from O1LDel infected mice to produce significantly more ($p < 0.05$) IL-2 than BMDC infected with O1LDel. A similar trend was seen with T cells from WR infected mice, but the difference was not significant. Compared to the 24 h supernatants, there were increased amounts of IL-2 detected at 48 h, and absorbance values did exceed 1 OD. Under these conditions, there was no evidence that O1L inhibits T cell response *in vivo* or recall antigen presentation *in vitro*.

We also used 10,000 and 50,000 BMDC infected with virus to incite a T lymphocyte response *in vitro*, since the Roper laboratory had previously detected IL-2 with 10,000 to 100,000 APC presenting antigen to T cells (Rehm et al., 2009; Rehm, Connor et al., 2010); and it was possible that cell crowding or large numbers of T cells absorbing IL-2 might reduce IL-2 detection. However, for this model, minimal to no IL-2 was detected when 10,000 BMDC were utilized (Figure 9), and T cell responses with 50,000 BMDC were lower than 100,000 BMDC (Figure 11A). Therefore, 100,000 BMDC were used in subsequent experiments. In antigen presentation assays using 50,000 BMDC, T cells from WR infected mice produced significantly more IL-2 than T cells from O1LDel infected mice, which did not indicate that O1L in VACV

inhibits T cell response (Figure 11A). BMDC infected with O1LDel did re-stimulate T cells to produce more IL-2 than WR infected BMDC in this experiment, but this difference was not reproducible.

The majority of antigen presentation experiments utilized an MOI of 1, which was previously found to be optimal in APC infections by the Roper lab (Rehm, Connor et al., 2010). APC were also infected at an MOI of 0.5 (Figures 12, 14A) and an MOI of 100 (Figure 11B) to look at MOI influence on O1L effects on antigen presentation and T cell response. At lower and higher MOIs, there were no data to suggest O1L is inhibitory. For BMDC infected with 100 pfu/cell, minimal IL-2 was detected, indicating very poor re-stimulation of the T cells (Figure 11B). Additionally, when a titration of 10,000 to 100,000 BMDC were infected and presenting viral peptides (Figures 8-11A), T cells from O1LDel infected mice were re-stimulated to produce similar or reduced amounts of IL-2 relative to wild type.

To further explore the effects of O1L on antigen presentation, a second mouse infection was completed in C57BL/6 female mice for 2-5 weeks, and antigen presentation assays were performed using unfractionated splenocytes or CD4⁺ T cells from infected mice. Figure 10 shows again that overall IL-2 production was similar between CD4⁺ T cells or splenocytes from WR and O1LDel infected mice. There was a small but significant difference in IL-2 production between splenocytes from WR and O1LDel infected mice when re-stimulated by O1LDel infected BMDC, and between CD4⁺ T lymphocytes from infected mice re-stimulated with wild type WR infected BMDC. In both cases, T cells from WR infected mice produced significantly more IL-2 than T cells from O1LDel infected mice, but there were no data to suggest that O1L in VACV inhibits T cell response.

For these experiments, BMDC were cultured for 8 days instead of 6 to determine if more differentiated BMDC would be able to release NO in response to antigen presentation. Similar to the A35 experiments, NO levels in the supernatants tested were equivalent to the media + Greiss negative control (data not shown).

Additionally, we performed antigen presentation experiments with splenocytes from infected mice without BMDC added. For mice infected for 5 weeks, splenocytes only were plated, and we relied on APC already present in the spleen to present viral peptides from viral infection. While splenocytes from O1LDel infected mice had increased IL-2 production compared to splenocytes harvested from wild type WR infected mice, there was not a significant difference in T cell response between the two groups (Figure 12). Minimal amounts of NO were detected in the supernatants compared to the negative control, and splenocytes from WR infected mice produced significantly more NO than splenocytes from O1LDel infected mice (Figure 13). However, there was no difference in NO between UN splenocytes and infected splenocytes, suggesting that the NO detected was not a result of antigen presentation with viral peptides.

Antigen Presentation to Transgenic T Cells

Antigen presentation assays were also performed with transgenic 2D2 mouse T cells. 2D2 mice are transgenic mice genetically modified to produce CD4⁺T cells that are only stimulated by a specific set of peptide antigens (MOG or NFM). Two experimental designs were used: 1) infecting unfractionated 2D2 splenocytes with WR or O1LDel viruses and pulsing with antigen or 2) infecting BMDC with WR or O1LDel, pulsing with antigen, and then adding 2D2 CD4⁺ T cells. We used MOG35-55 or NFM13-37 antigens at different concentrations to re-stimulate the T cells as well as various numbers of splenocytes or T cells. Compared to the negative control (no antigen added), we were able to stimulate the 2D2 T cells to release IL-2,

and there was a reduction in transgenic T lymphocyte response in wells with VACV infected cells compared to UN (Figure 14). O1LDel infected splenocytes produced slightly more IL-2 relative to WR infected splenocytes, but the difference was not significant (Figure 14A). In Figure 14B, T cells stimulated by O1LDel infected BMDC produced significantly less IL-2 compared to T cells stimulated by WR infected BMDC when MOG was used as the antigen. A similar trend was seen for wells with infected BMDC pulsed with NFM. While sometimes the WR infected cells showed higher or lower IL-2 production compared to O1LDel infected cells, there was not a pattern of inhibition. Based on multiple antigen presentation assays with various experimental conditions, there is no evidence to indicate that VACV O1L inhibits antigen presentation or T cell response.

O1L and Anti-viral Antibody Production

While O1L does not appear to affect antigen presentation or T cell response, previous studies by the Roper laboratory have suggested O1L may inhibit anti-viral antibody production. To study O1L effects on this process, blood was harvested from C57BL/6 male mice infected with WR, O1LDel, or mock infected with PBS for the antigen presentation assays at 1, 2, 3, and 4 weeks after infection. Sera were then tested using a virus specific ELISA. We found that there was not a significant difference in anti-viral antibody production between mice infected with WR or O1LDel viruses for 1 to 4 weeks (Figure 15). Compared to the negative control (sera from mice mock infected with PBS), mice infected with virus produced increased amounts of antibody indicating a VACV specific response.

We repeated this experiment in female C57BL/6 mice by infecting with WR, O1LDel, or PBS and harvesting sera 3 weeks post infection. To ensure infection occurred, viral dose was increased from 800 pfu/mouse to 1×10^4 pfu/mouse. Mouse weight was monitored the first few

days post infection due to the augmented viral dose, and several of the mice lost weight, indicating the infection was successful. Anti-viral antibody production for mice infected with WR versus O1LDel for 3 weeks was similar for all wells except for at dilution 2 (2.2 ul of sera added), where the difference was small but significant (Figure 16).

Taken together, the ELISA results suggest O1L does not affect the production of anti-viral antibody. However, a previous student from our laboratory showed O1L inhibits antibody production at 7 days post infection in BALB/c mice. Therefore, we repeated the mouse infection in BALB/c mice carefully following procedures used by the previous student and measured the amount of antibody present in the sera. Once again, O1LDel infected mice had similar antibody production relative to WR infected mice (Figure 17). In fact, at 7 days post infection, the amount of anti-viral antibody present in the infected mice was comparable to the PBS control.

A fourth mouse infection was performed in C57BL/6 female mice for 2-5 weeks to confirm the findings. As seen previously in Figures 15-17, anti-viral antibody production in O1LDel infected mice was similar to wild type WR infected mice for all weeks tested, and no statistically significant difference was found (Figure 18). At week 4, one WR infected mouse had a very low antibody response, and we suspect it was poorly infected. However, it did not pass the exclusion rule, so it decreased the average antibody production of the group. Based on the data collected from multiple mouse infections with different infection lengths, viral doses, and mouse strains, there is no evidence that VACV O1L inhibits anti-viral antibody production *in vivo*.

O1L and Cell killing

One manuscript reported that O1L affects the Raf/MEK/ERK intracellular pathway which is involved in several cellular functions including cell proliferation and growth and enhanced viral

replication (Schweneker et al., 2012). The O1LDel virus was analyzed for replication and spread by performing viral plaque assays. Confluent monolayers of BS-C-1 cells were infected with O1LDel or WR and plaque formation was observed. For the majority of the time, O1LDel plaques were similar in size and shape to wild type WR (Figure 19A). However, in some cases, O1LDel plaques appeared smaller than WR plaques (Figure 19B). Upon higher magnification, it was revealed that O1LDel plaques had similar diameters to wild type, but there was less cell clearance in the center of the plaques (Figure 20). This phenomenon was previously seen by the Roper laboratory as well as others (Schweneker et al., 2012). A possible explanation for this disparity is that O1L affects cell killing, which may be related to the role of the Raf/MEK/ERK pathway in cell survival and death. We investigated this hypothesis using BMDC that were infected with WR or O1LDel for approximately 48 h, and then cellular metabolism was measured using an MTS assay. BMDC infected with WR or O1LDel had significantly reduced metabolism relative to UN (Figure 21). However, there was no difference in metabolism between cells infected with WR versus O1LDel. The cell killing experiments were repeated using CTLL and splenocytes. VACV infection reduced cell metabolism in CTLL and splenocytes (Figure 22). While O1LDel infected splenocytes had comparable metabolism to WR infected splenocytes, there was a small but significant difference between infected CTLL, with WR infected CTLL having slightly increased metabolism compared to O1LDel infected cells. Based on the data collected, there is no evidence to suggest O1L affects cell killing in BMDC, CTLL, or splenocytes under the conditions tested.

Effect of O1L on Integrin Expression

Another potential function of O1L in the Raf/MEK/ERK pathway is influencing cell migration, which was supported by preliminary data from the Roper laboratory. Therefore, we looked at

O1L and integrin expression. As previously described, integrins are an important part of leukocyte migration, also known as diapedesis, and cell-cell interactions. To examine the effects of O1L on integrin expression, BMDC were infected with WR or O1L Δ Del and stained with a primary antibody for CD11a, CD18, or CD29 integrins, followed by a secondary antibody to measure protein levels by flow cytometry. Compared to the negative control (BMDC stained with secondary antibody only), the sample peaks were shifted to the right, indicating that the integrins were expressed on BMDC. However, the levels of CD11a, CD18, and CD29 detected were similar between UN, WR, and O1L Δ Del infected BMDC (Figure 23). These data provide no evidence that VACV infection or the O1L protein affects expression of the 3 integrins tested on BMDC.

Cytokine Production

The Raf/MEK/ERK pathway is also known to impact cell differentiation, which may affect cytokine production. While O1L did not appear to have an effect on IL-2 production during antigen presentation assays, it was possible O1L alters the production of other cytokines that are released in response to antigen presentation. A cytokine bead kit was used to measure levels of IL-2, IL-4, IL-6, IFN- γ , TNF, IL-17A, and IL-10 in supernatant samples from antigen presentation assays between BMDC and splenocytes from infected mice (3 or 4 weeks) (Figure 10A: Week 3 IL-2 data). Compared to the CTLL IL-2 bioassay that quantifies biologically active IL-2, the bead kit measures the total amount of IL-2 present. The different cytokines were distinguished using beads with unique mean fluorescent intensities that were coated with antibody for each of the cytokines. Splenocytes from mice infected for 3 weeks were re-stimulated to release IL-2, IFN- γ , and IL-6 as evidenced by the rightward shift of the WR and O1L Δ Del peaks relative to UN (Figure 24). Week 4 samples showed similar cytokine production

to week 3 (data not shown). However, in both weeks minimal to no IL-4, IL-10, and IL-17A were detected (data not shown). Of the cytokines measured, there was no difference in cytokine production between splenocytes from O1L^{Del} infected mice versus splenocytes from wild type WR infected mice, providing no evidence that O1L affects the *in vitro* production of the cytokines tested.

Effect of O1L on Innate Immunity

Since there was no evidence to suggest that O1L affects the adaptive immune response, we investigated the hypothesis that O1L functions in the innate immune system by using hemolymph from waxworms. In one experiment, BS-C-1 monolayers in a 96 well plate were pre-incubated with 5 ul hemolymph for 10 min and infected with virus, or varying concentrations of hemolymph were incubated with virus for 10 min and added to cells. The samples were harvested approximately 21 hpi, and titers were measured using viral plaque assays. Compared to monolayers infected with virus only, the addition of hemolymph resulted in a large reduction in pfu (Figure 25). Wells with virus + hemolymph had pfu comparable to the input virus which was 100 pfu/well, while virus only infected cells produced at least 100 times more pfu at ~21 hpi. Virus incubated with increasing amounts of hemolymph or cells that were pre-incubated with hemolymph before viral infection showed an even greater reduction in plaque numbers (data not shown). Because both viruses had similar plaque numbers when hemolymph was added, there is no evidence to indicate that VACV O1L inhibits the anti-viral effects of the worm hemolymph or the innate immune response under these experimental conditions. However, only one experiment was performed, and the concentration of hemolymph caused a layer of granular debris over the cells.

CHAPTER 4: FIGURES

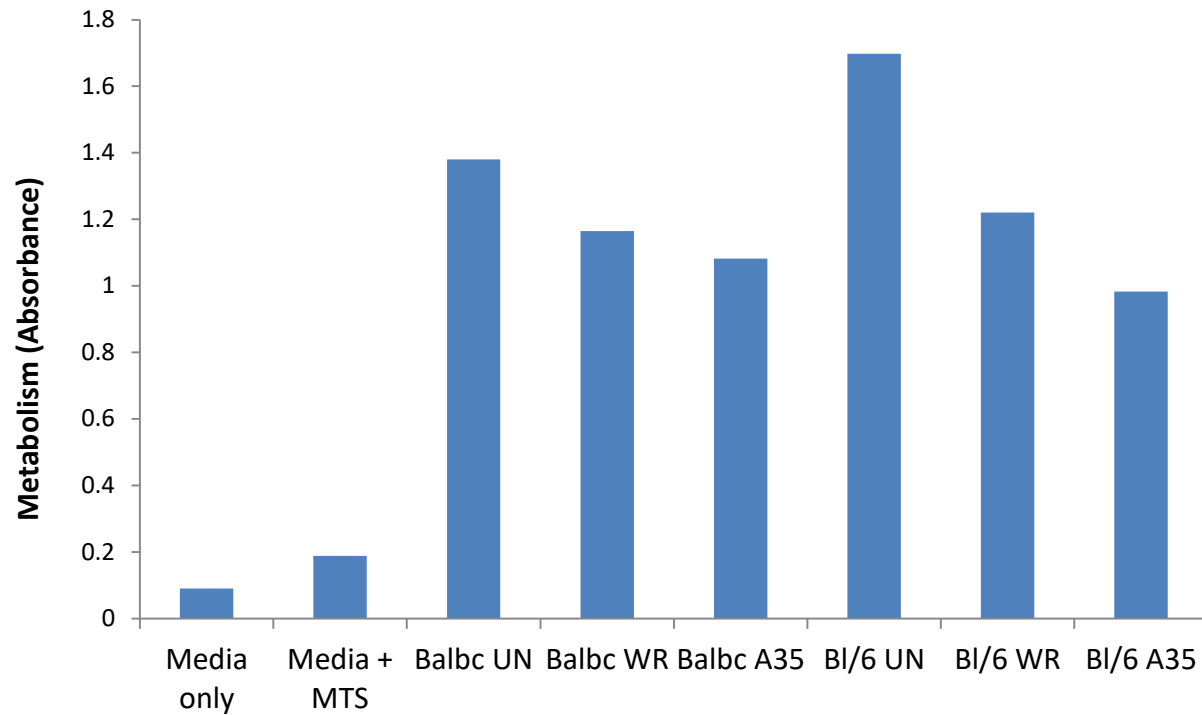


Figure 1. VACV cell killing in BMDC. BALB/c and C57BL/6 BMDC (one well) were UN or infected with WR or A35Del viruses for ~48 h. Then, MTS was added to measure cellular metabolism. Media only and media + MTS were used as negative controls. This experiment was performed in conjunction with the one step growth curve. There was no evidence that A35 in WR increased cell killing, so further experiments were not performed to determine significance.

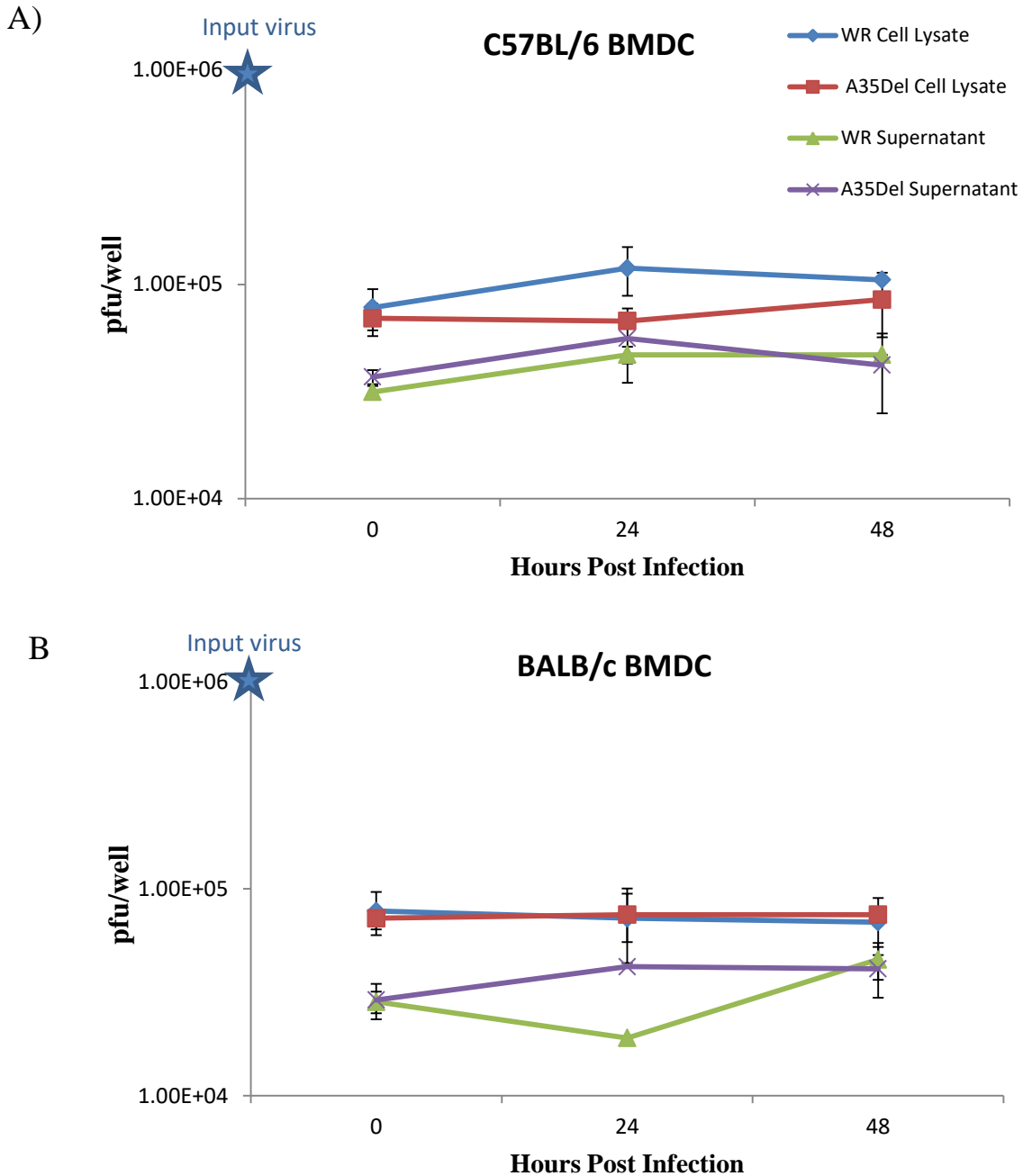


Figure 2. VACV replication in BMDC. A) C57BL/6 and B) BALB/c BMDC were infected with WR or A35Del viruses at an MOI = 10 for 1.5 h. After a wash to remove excess virus, cells were plated and incubated at 37°C. At each specific time point, cell lysate and supernatants were harvested in duplicate to determine virus titer using viral plaque assays. One step growth curves were performed twice in each mouse strain. The growth curve depicted shows the results from the 2nd experiment which is representative of the overall results, and the data represent the averages (\pm SD). SD at 24 h for BALB/c supernatants was 0. Virus produced never exceeded input.

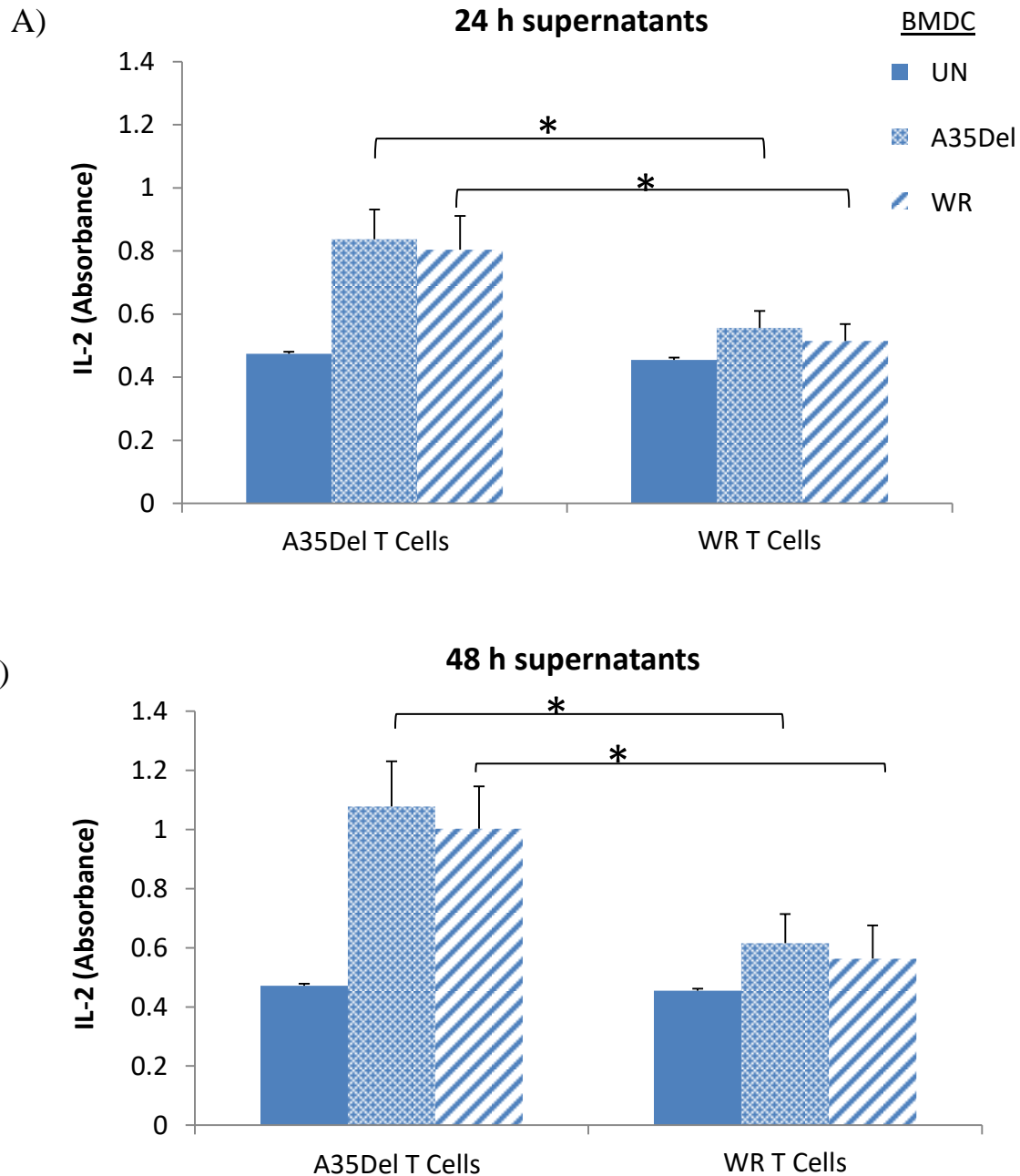


Figure 3. IL-2 production as a result of antigen presentation. Mice (n=5) were infected for 4 weeks with WR or A35Del viruses. BMDC were UN or infected for 4-5 h with WR or A35Del, and then CD4⁺ T cells were isolated from infected mice and combined in a 96 well plate with the BMDC. Supernatants were harvested and tested for cytokine production. CTLL IL-2 bioassays were performed using supernatants taken at approximately A) 24 h and B) 48 h. Data show the averages (\pm SEM) and were analyzed using two-way and one-way ANOVA, followed by post hoc unpaired two-tailed Student's T-Tests. *p<0.05.

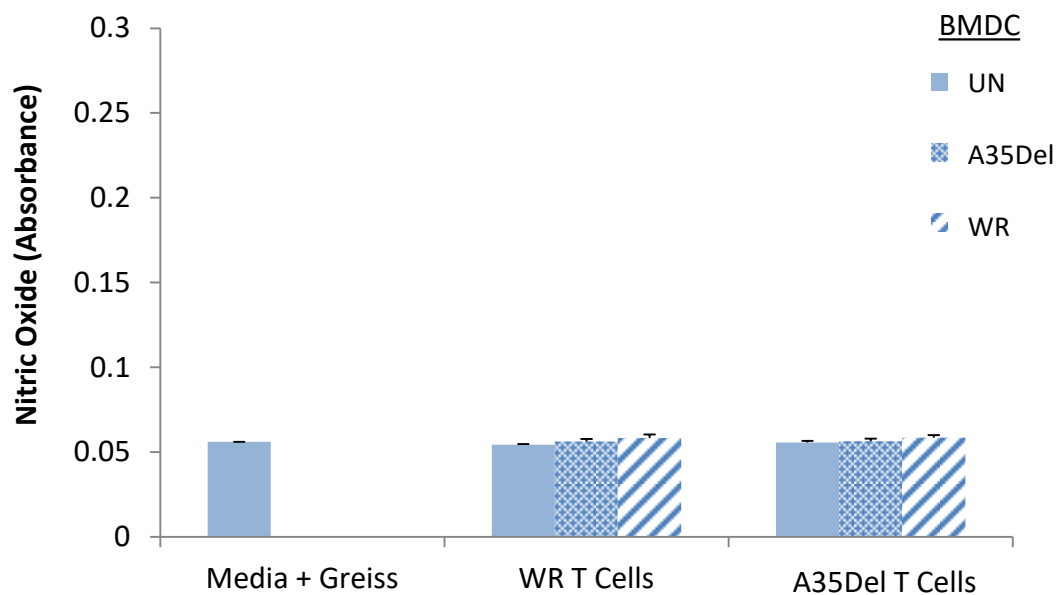


Figure 4. NO production as a result of antigen presentation. Supernatants taken from antigen presentation experiments were tested for NO production using a Greiss Assay. NED and sulfanilamide solutions were mixed together and added to sample wells. Absorbances were measured using a plate reader set to 550nm. Media and Greiss were utilized as the negative control. Diluted nitrite standard served as the positive control and had an absorbance of 0.656. Data show the averages of 5 samples (\pm SEM).

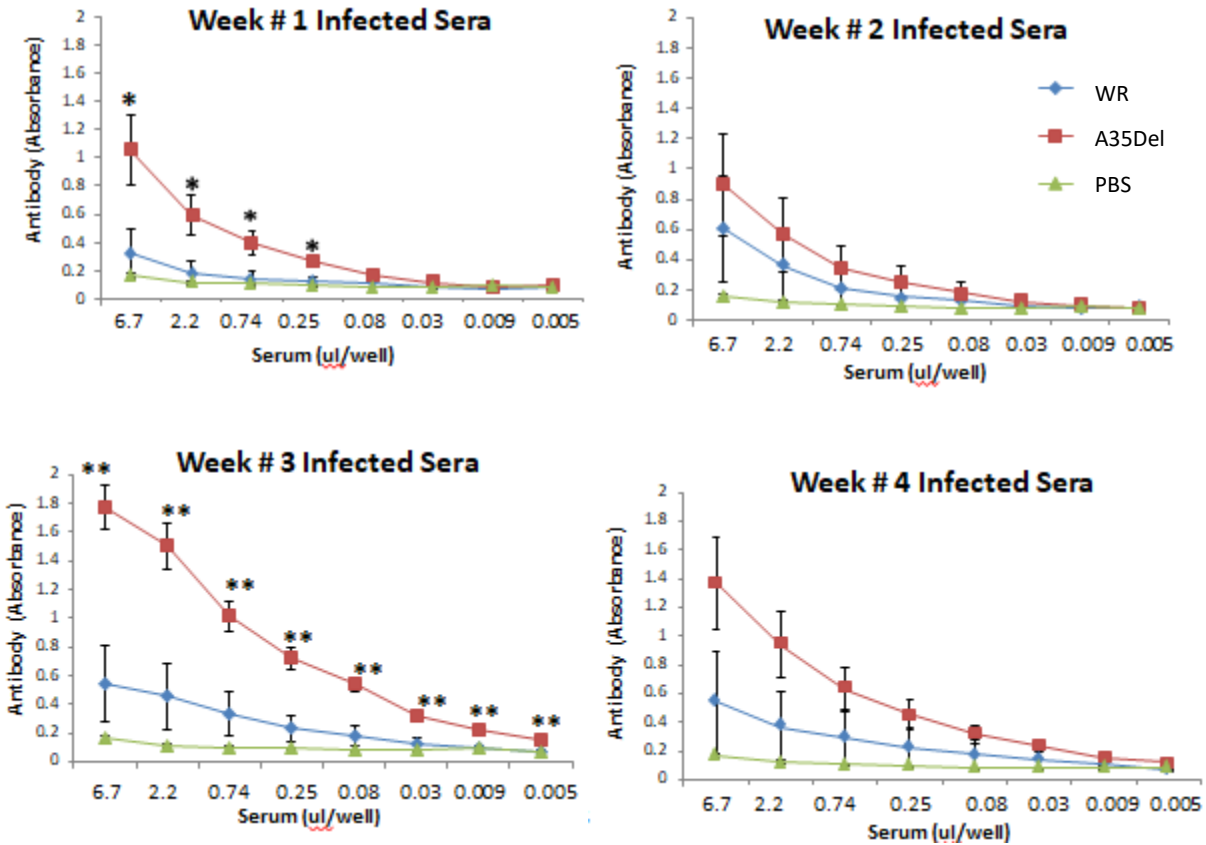


Figure 5. VACV specific antibody production. Mice (n=5) were infected with WR, A35Del virus, or mock infected with PBS for 1-4 weeks. Blood was harvested from infected mice, and sera were isolated to be tested via ELISA for anti-viral antibody production. The PBS infected mice served as a negative control. Unpaired two-tailed Student's T-Tests were used for statistical analysis. Data show averages (\pm SEM).

* $p < 0.05$, ** $p < 0.01$.

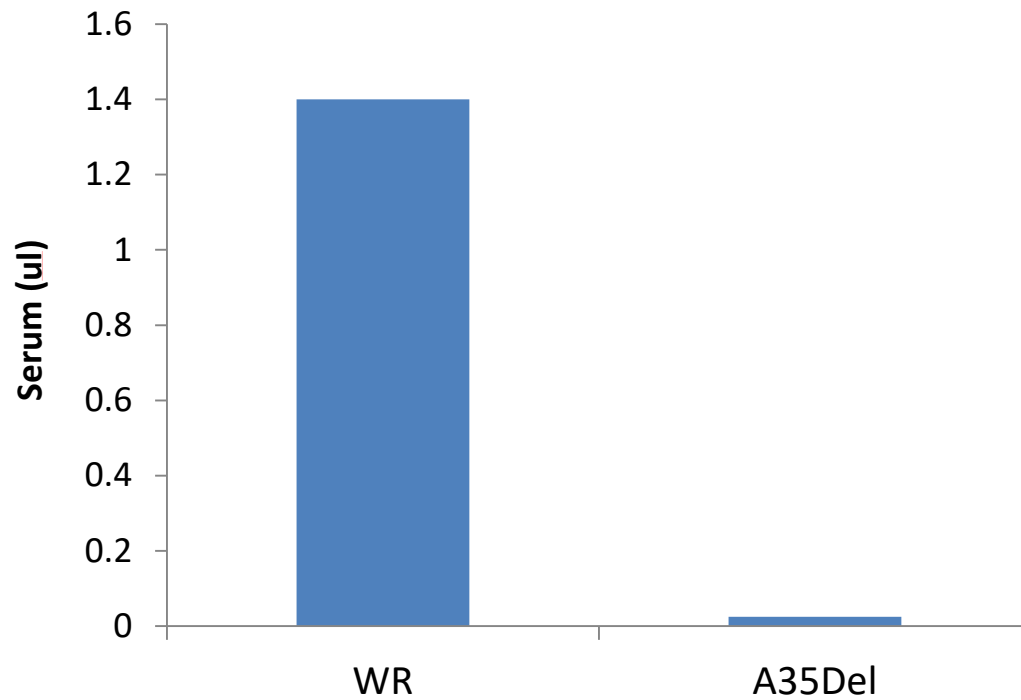


Figure 6. Comparison of sera from WR and A35Del infected mice. The ELISA data from Figure 5 were further analyzed by finding the line of best fit for the dilution curves from week #3 infected sera, and the amount of sera from WR or A35Del infected mice necessary to reach an absorbance of 0.4 OD was calculated. It takes over 50 times more sera from mice infected with WR to reach the same absorbance as sera from A35Del infected mice.

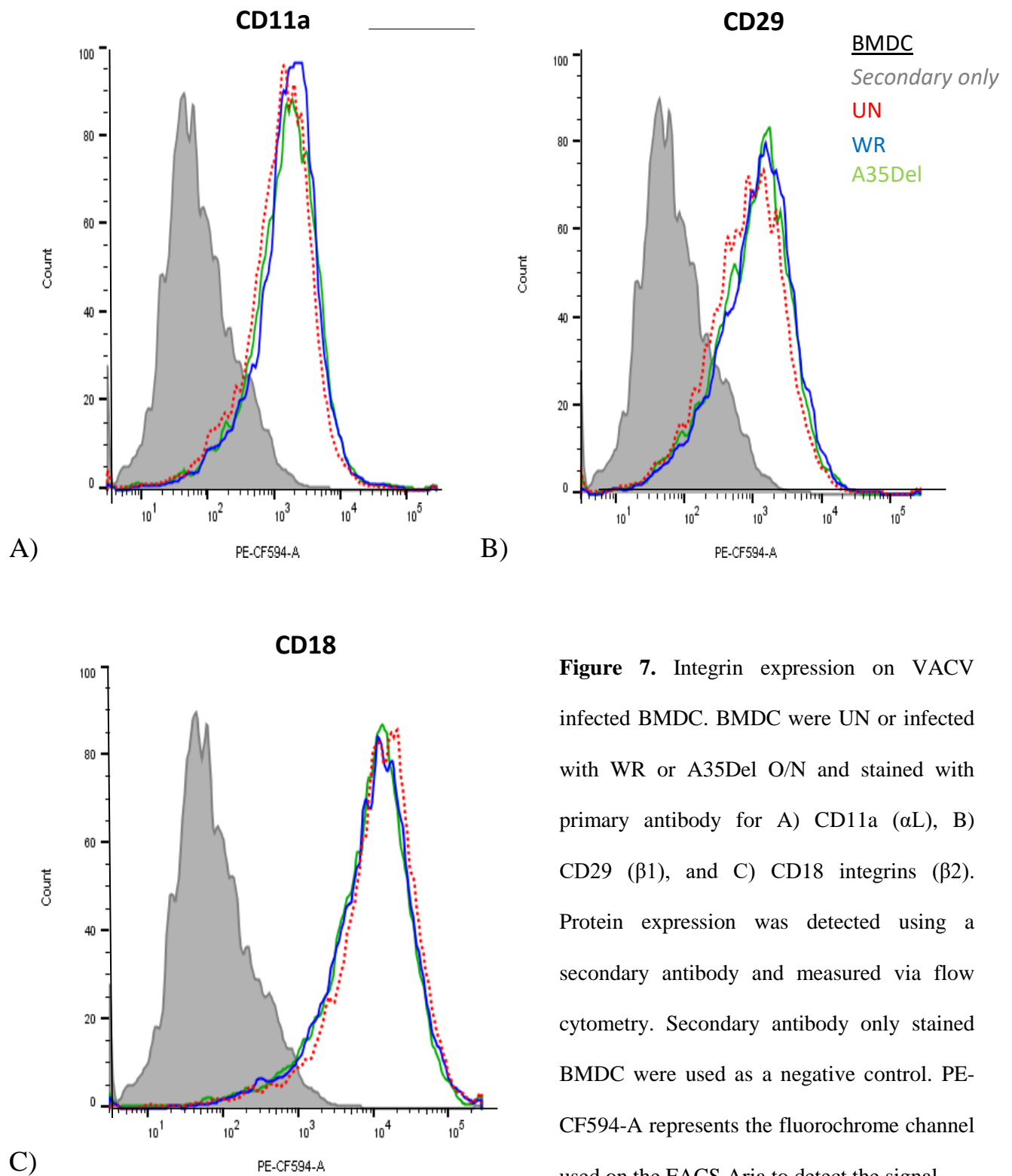


Figure 7. Integrin expression on VACV infected BMDC. BMDC were UN or infected with WR or A35Del O/N and stained with primary antibody for A) CD11a (α L), B) CD29 (β 1), and C) CD18 integrins (β 2). Protein expression was detected using a secondary antibody and measured via flow cytometry. Secondary antibody only stained BMDC were used as a negative control. PE-CF594-A represents the fluorochrome channel used on the FACS Aria to detect the signal.

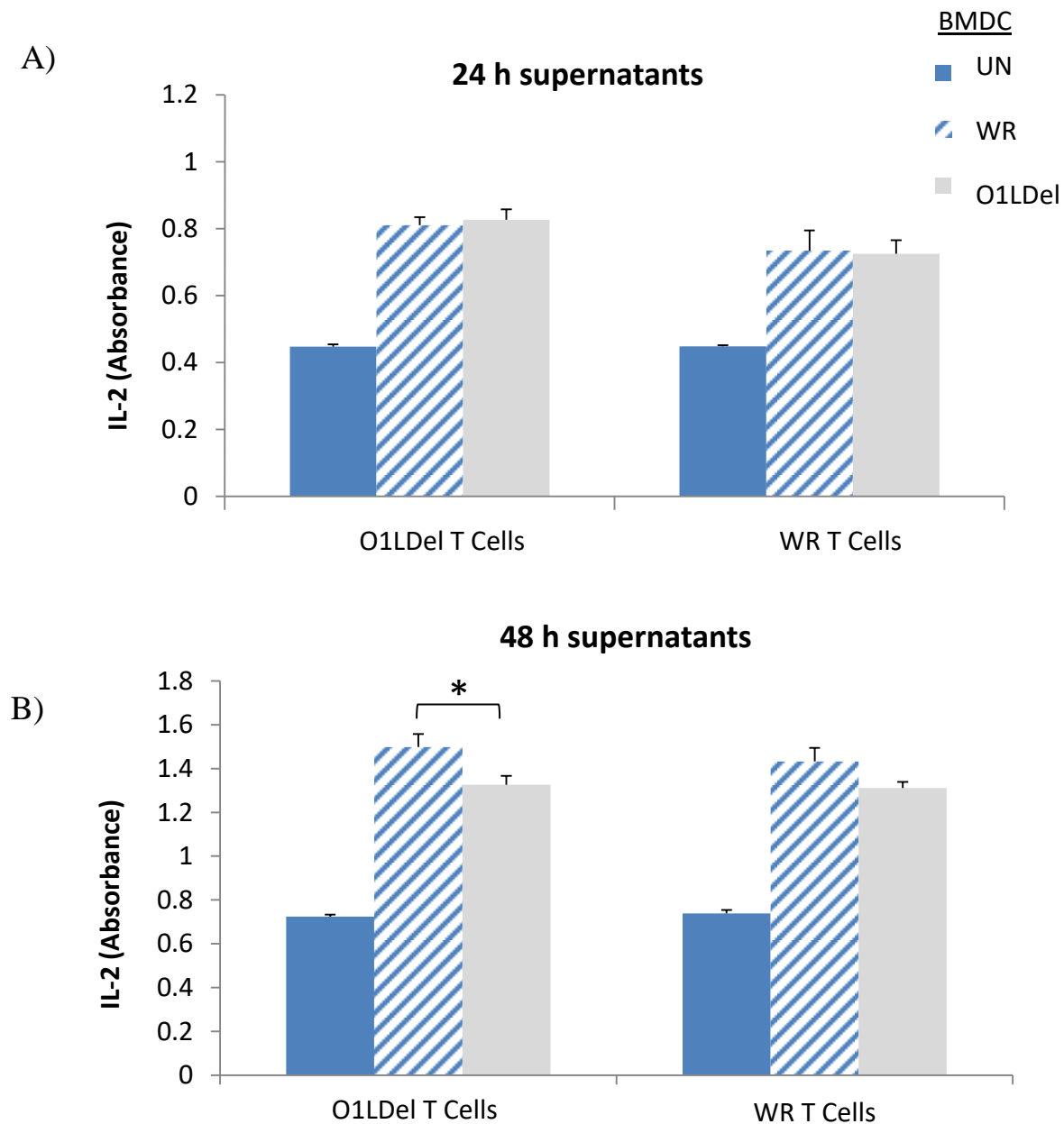


Figure 8. IL-2 produced from antigen presentation. Mice (n=5) were infected for 4 weeks with WR or O1LDe1 viruses. BMDC were UN or infected for 4-5 h with WR or O1LDe1, and then CD4⁺ T cells were isolated from infected mice and combined in a 96 well plate with the BMDC. Supernatants were harvested and tested for cytokine production. CTLL IL-2 bioassays were performed using supernatants taken at approximately A) 24 h and B) 48 h. Data show the averages (\pm SEM). Statistics performed via two-way ANOVA, followed by one-way ANOVA and post hoc unpaired two-tailed Student's T-Tests. *p<0.05.

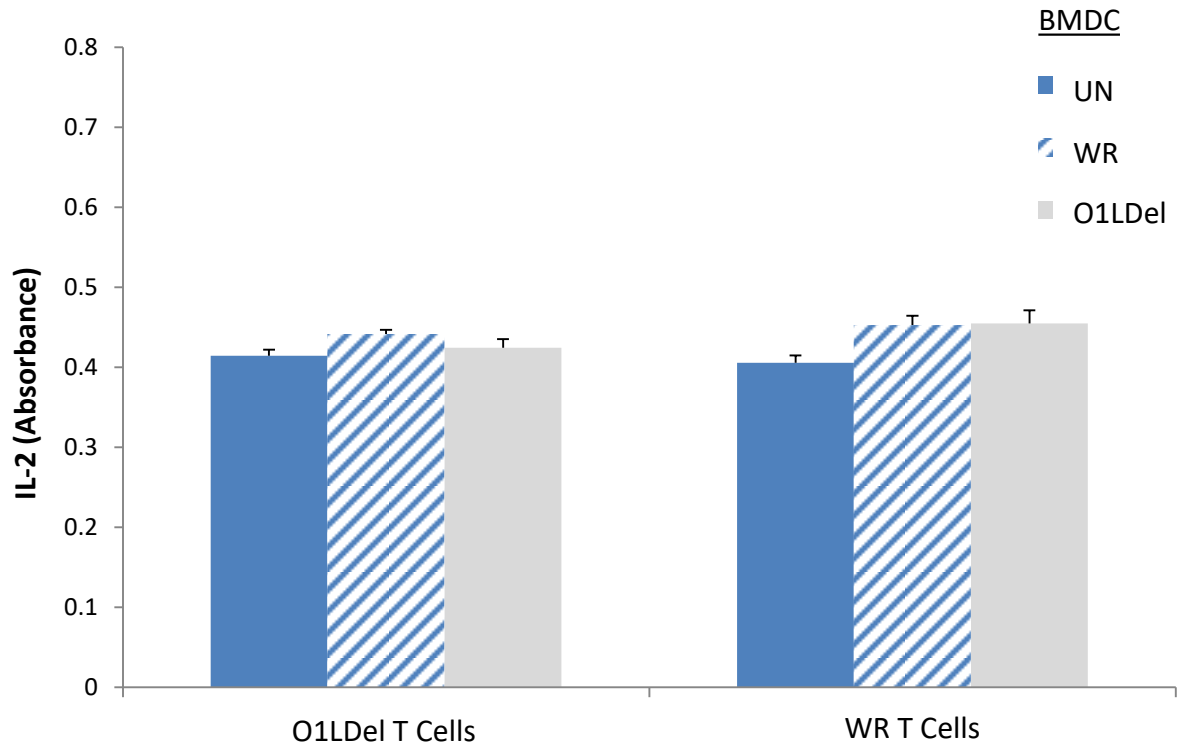


Figure 9. IL-2 produced from antigen presentation with 10,000 BMDC. Mice (n=5) were infected for 4 weeks with WR or O1LDeI. 10,000 BMDC were UN or infected for 4-5 h with WR or O1LDeI, and then CD4⁺ T cells were isolated from infected mice and combined in a 96 well plate with the BMDC. Supernatants taken approximately 24 h post addition of T cells were tested for IL-2 production using a CTLL IL-2 bioassay. Data show the averages (\pm SEM).

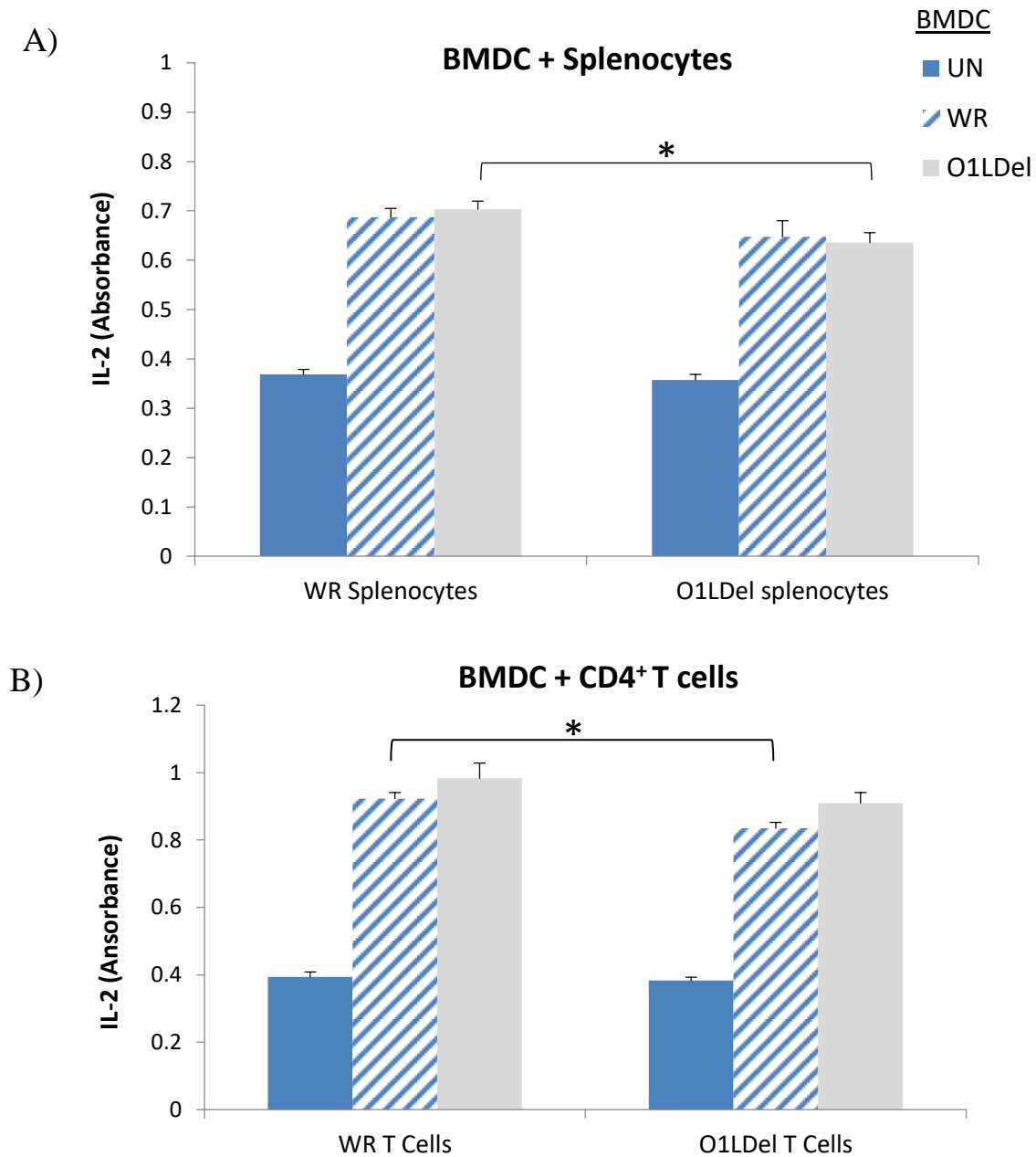
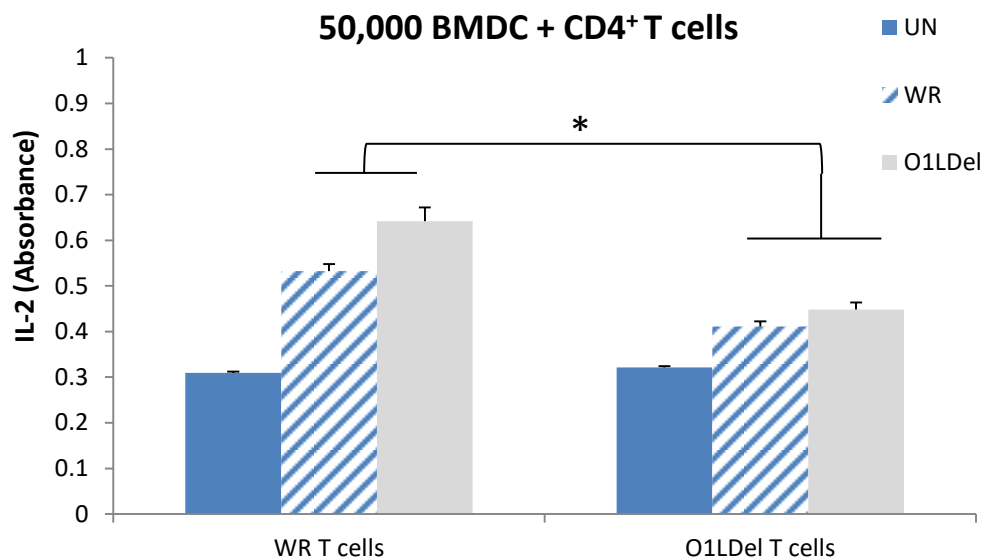


Figure 10. IL-2 production as a result of antigen presentation with splenocytes or CD4⁺ T cells. Mice (n= 3) were infected for 3 weeks with WR or O1LDel. 100,000 BMDC were UN or infected for 4-5 h with WR or O1LDel, and then A) splenocytes or B) CD4⁺ T cells were isolated from infected mice and combined in a 96 well plate with the BMDC. Supernatants were harvested at approximately 48 h and tested for IL-2 using a CTLL IL-2 bioassay. Data show the averages (\pm SEM). Statistics performed using two-way ANOVA, one-way ANOVA, and post hoc unpaired two-tailed Student's T-Tests. *p<0.05.

A)



B)

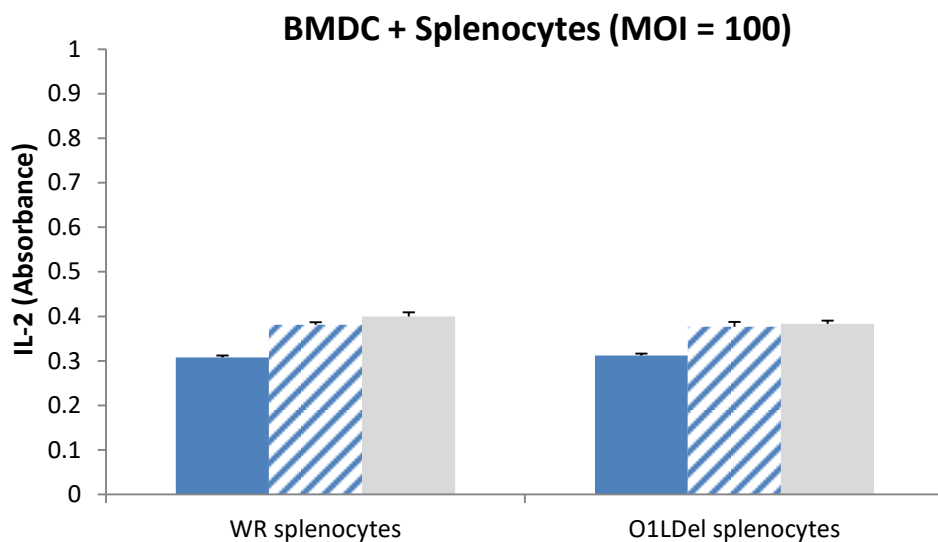


Figure 11. IL-2 production from antigen presentation with different BMDC number or MOI. Mice (n=4) were infected for 2 weeks with WR or O1LDel. A) 50,000 BMDC were UN or infected with WR or O1LDel at an MOI=1 for 4-5 h. CD4⁺ T cells were isolated from infected mice and combined in a 96 well plate with BMDC. B) 100,000 BMDC were UN or infected with WR or O1LDel at an MOI=100 for approximately 1 h, and unfractionated splenocytes from infected mice were added to the BMDC. Supernatant samples were taken at ~48 h and tested for IL-2 using CTLL IL-2 bioassay. Data show the averages (\pm SEM). Statistics performed using two-way and one-way ANOVA and post hoc unpaired two-tailed Student's T-Tests. *p<0.01.

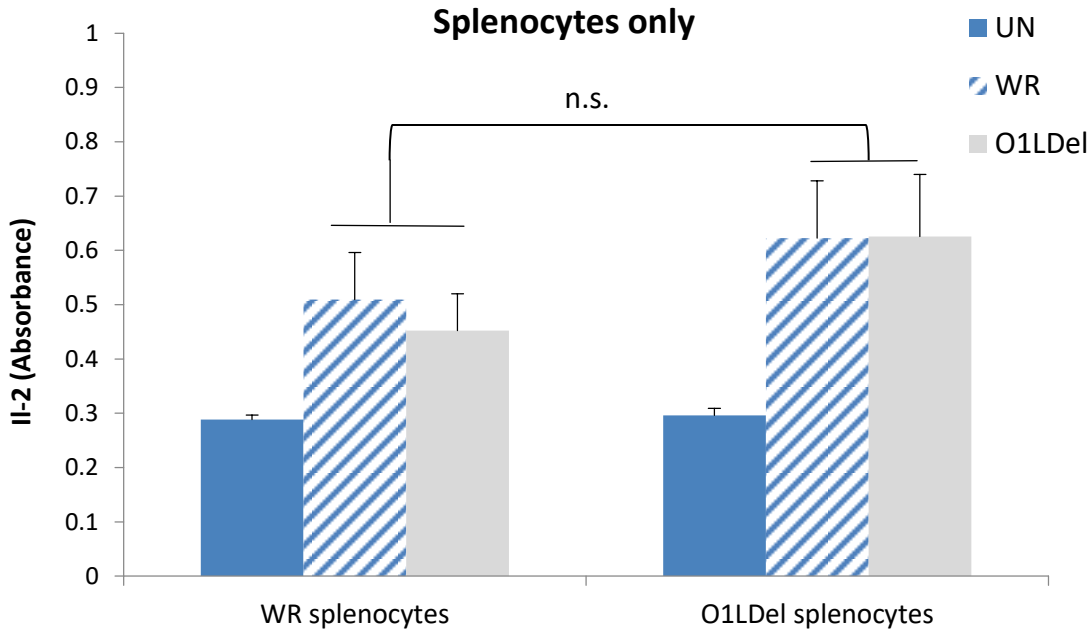


Figure 12. IL-2 production from antigen presentation with splenocytes only. Mice (n=5) were infected with WR or O1LDeI for 5 weeks, and splenocytes were harvested and UN or re-stimulated with WR or O1LDeI. Supernatants were taken at approximately 48 h and tested for IL-2 with an IL-2 bioassay. Data show the averages (\pm SEM). There was no statistically significant difference between WR and O1LDeI splenocytes. Statistics calculated using two-way ANOVA, one-way ANOVA, and post hoc unpaired two-tailed Student's T-Test's.

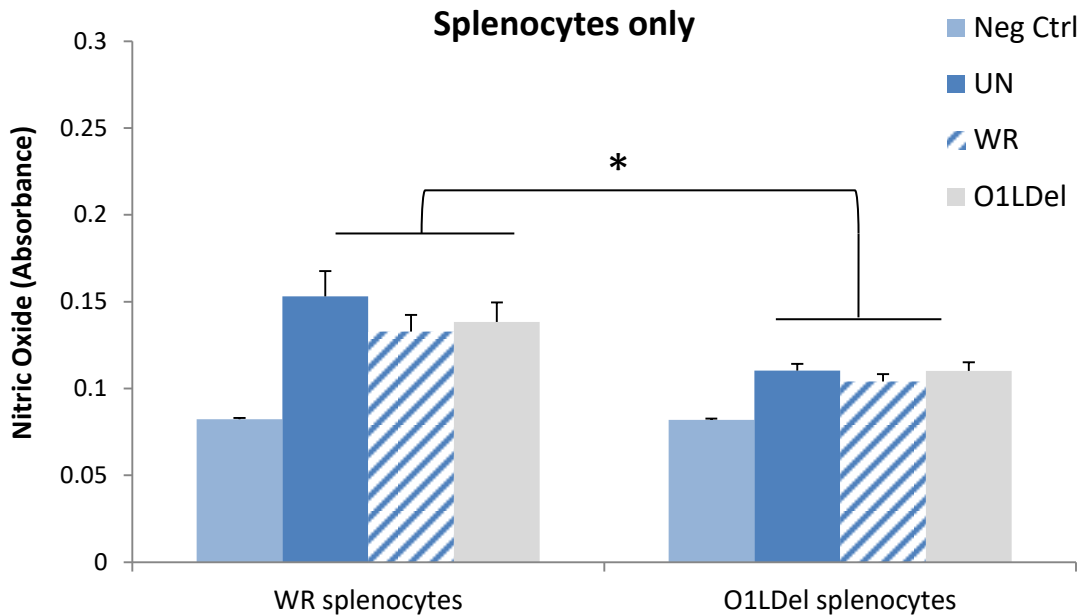


Figure 13. NO production as a result of antigen presentation with splenocytes only. Mice (n=5) were infected with WR or O1LDeI for 5 weeks. Splenocytes were harvested from infected mice and UN or re-stimulated with WR or O1LDeI. Supernatants were taken at approximately 48 h and tested for NO with a Greiss Assay. Media + Greiss were used as the negative control. Diluted nitrite standard served as the positive control and had an absorbance of 0.334. Data show the averages (+ SEM). Statistics performed using two-way ANOVA, one-way ANOVA, and post hoc unpaired two-tailed Student's T-Test's. *p<0.05.

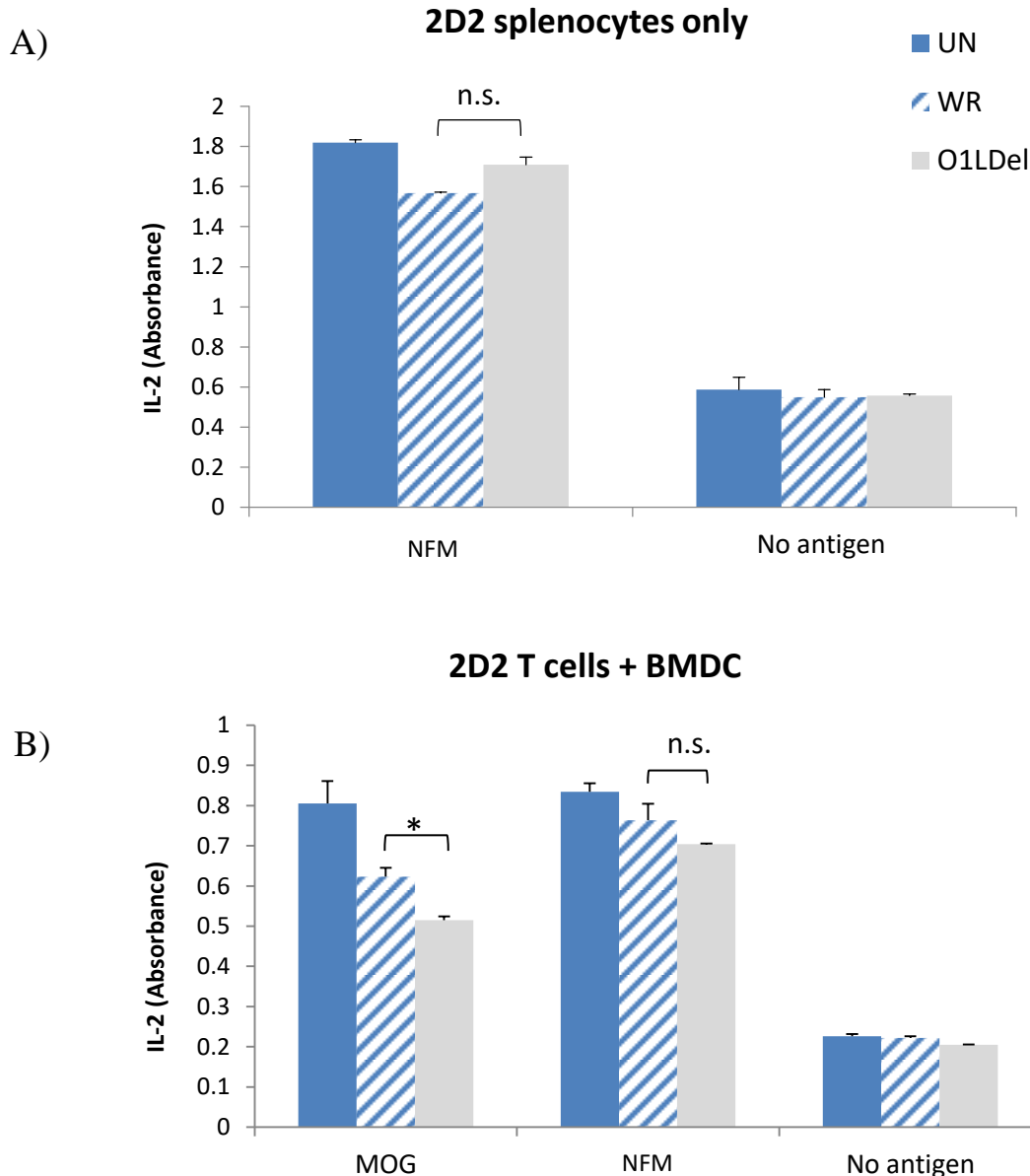


Figure 14. IL-2 production from antigen presentation to transgenic T cells. A) 2D2 splenocytes were either UN or infected with WR or O1LDeI at an MOI= 0.5 for ~3 h. Then, cells were incubated with 320 nM of NFM13-37, and supernatants were harvested at ~43 h to be tested for IL-2. B) 100,000 BMDC were either UN or infected with WR or O1LDeI at an MOI=1 for 4-5 h then pulsed with 5 uM of MOG35-55 or 500 nM of NFM13-37 for 30 min. 2D2 T cells were added, and supernatants were taken after approximately 24 h to be tested for IL-2. Data represent the average of 3 wells (\pm SEM). Two-way ANOVA and one-way ANOVA, followed by post hoc unpaired two-tailed Student's T-Tests were used for statistical analysis. * $p < 0.05$.

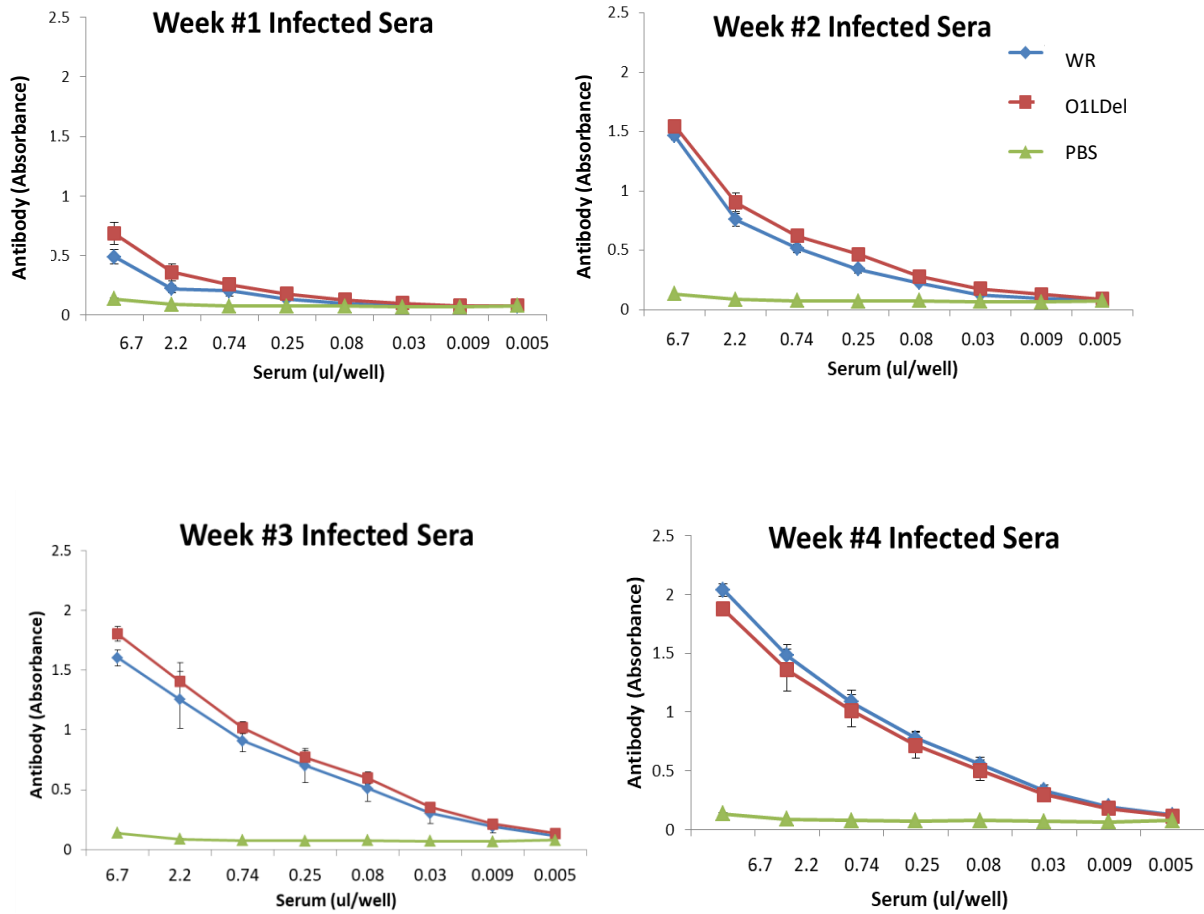


Figure 15. VACV specific antibody response. Mice (n=5) were infected with WR, O1LDeI, or mock infected with PBS for 1-4 weeks. Blood was harvested, and sera were isolated to be tested for anti-viral antibody production with ELISA. Several mice were suspected to be UN because their antibody response was not above that of PBS infected mice and were omitted using an exclusion rule. Data show the averages (\pm SEM). There was no significant difference in anti-viral antibody between sera from WR or O1LDeI infected mice via unpaired two-tailed Student's T-Tests.

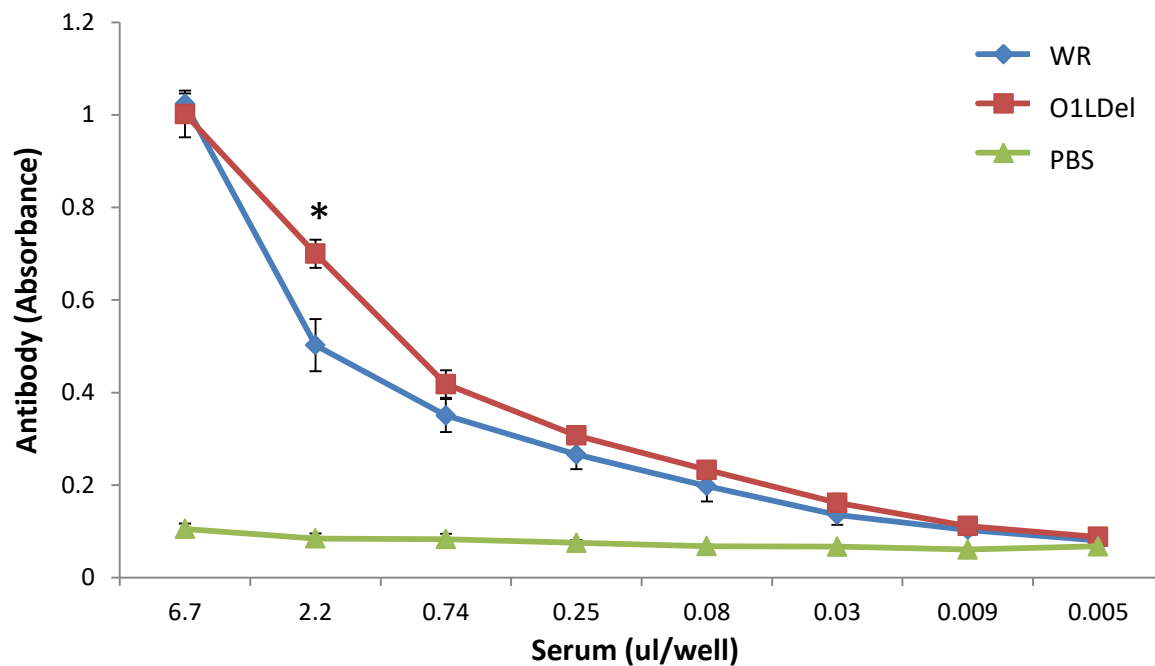


Figure 16. VACV specific antibody production (repeat week 3). C57BL/6 female mice (n=3) were infected with WR, O1LDeI, or mock infected with PBS for 3 weeks. Infectious dose was increased from 800 pfu to 1×10^4 pfu to ensure mice became infected. Sera were harvested and tested for anti-viral antibody production using ELISA. Unpaired two-tailed Student's T-Tests were utilized for statistical analysis. Data represent the averages (\pm SEM). * $p < 0.05$.

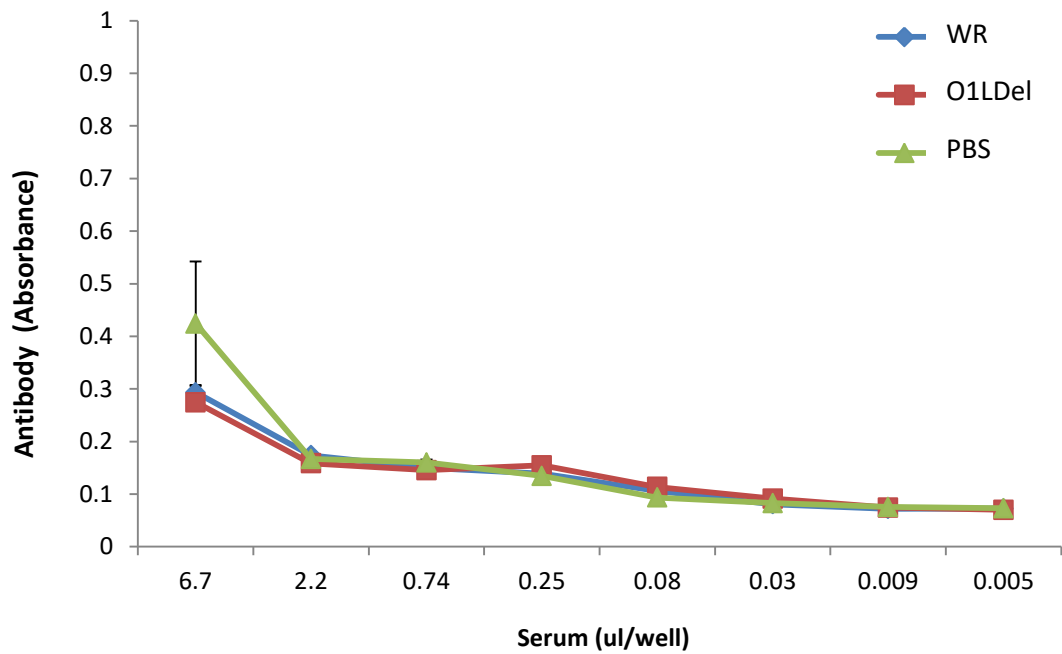


Figure 17. Anti-viral antibody production in BALB/c mice. BALB/c (n=5) mice were infected with WR, O1LDeI, or mock infected with PBS for 7 days. Blood was harvested, and sera were isolated to be tested for anti-viral antibody production with ELISA. PBS mock infected mice were used as a negative control. Data represent the averages (\pm SEM).

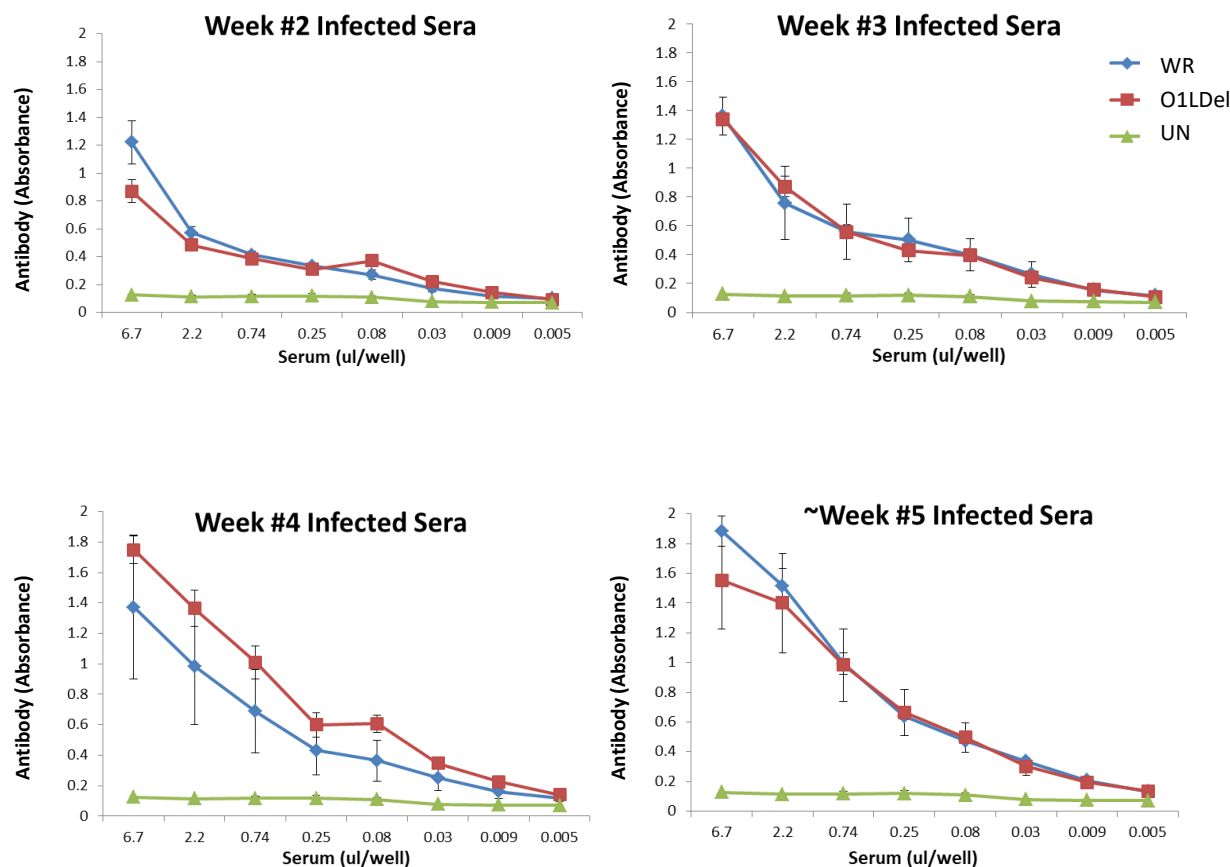


Figure 18. Anti-viral antibody production in mice infected 2 to 5 weeks. Mice ($n = 3-5$) were infected with WR, O1LDeI, or UN for 2-5 weeks. Blood was harvested, and sera were isolated to be tested for anti-viral antibody via ELISA. Data show the averages (\pm SEM). In week 4, there was 1 mouse with low antibody, but it did not pass the exclusion rule and was kept in the data set. No significant difference in antibody production between mice infected with WR and O1LDeI, calculated using unpaired two-tailed Student's T-Tests.

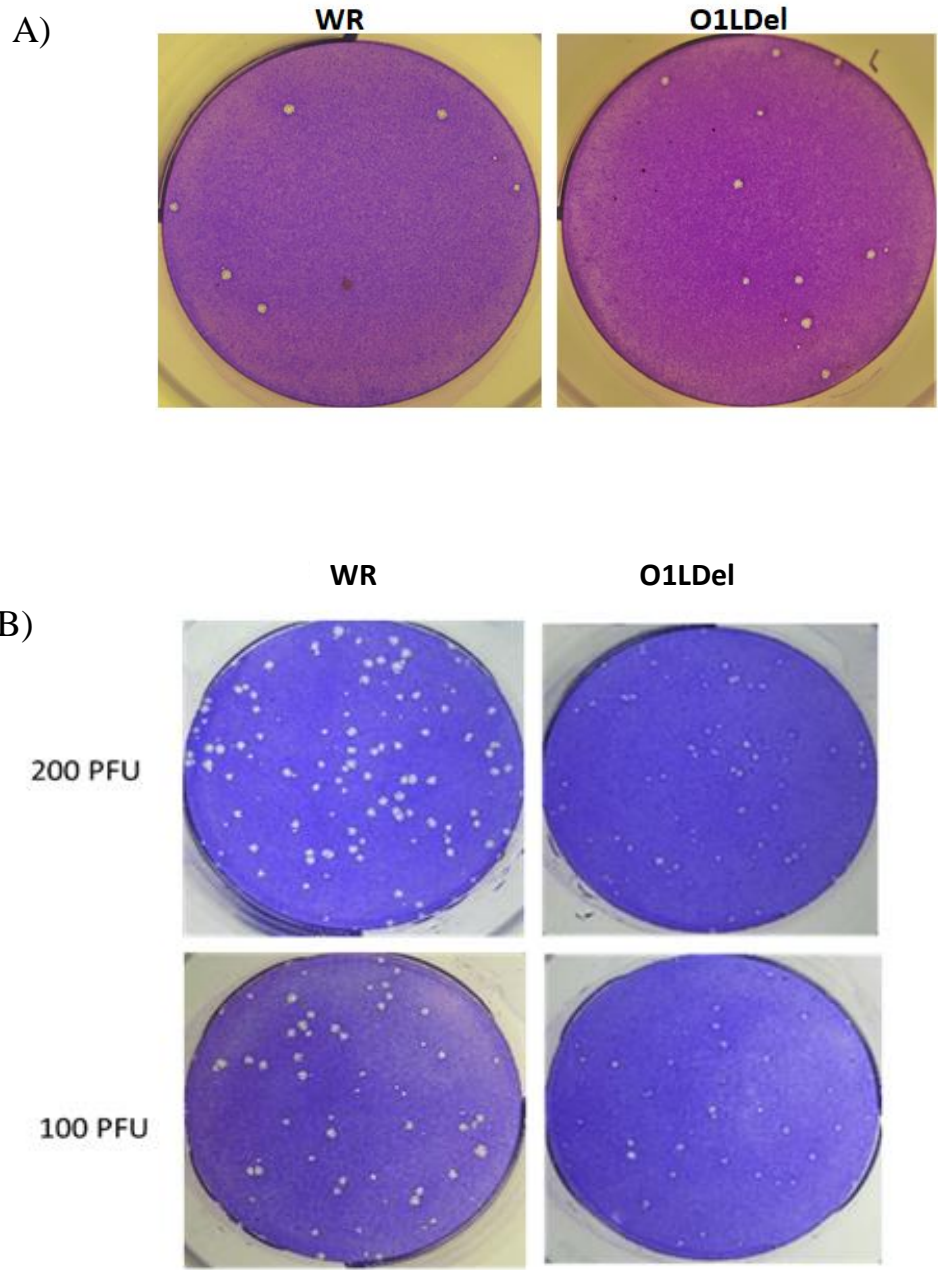


Figure 19. VACV replication and spread. Confluent BS-C-1 monolayers were grown in 6 well plates, infected with WR or O1LDel for 40-48 h, and stained with crystal violet to measure viral titers. A) In most cases the O1LDel plaques had similar morphology to WR plaques. B) However in a few cases, O1LDel plaques appeared smaller than wild type WR plaques.

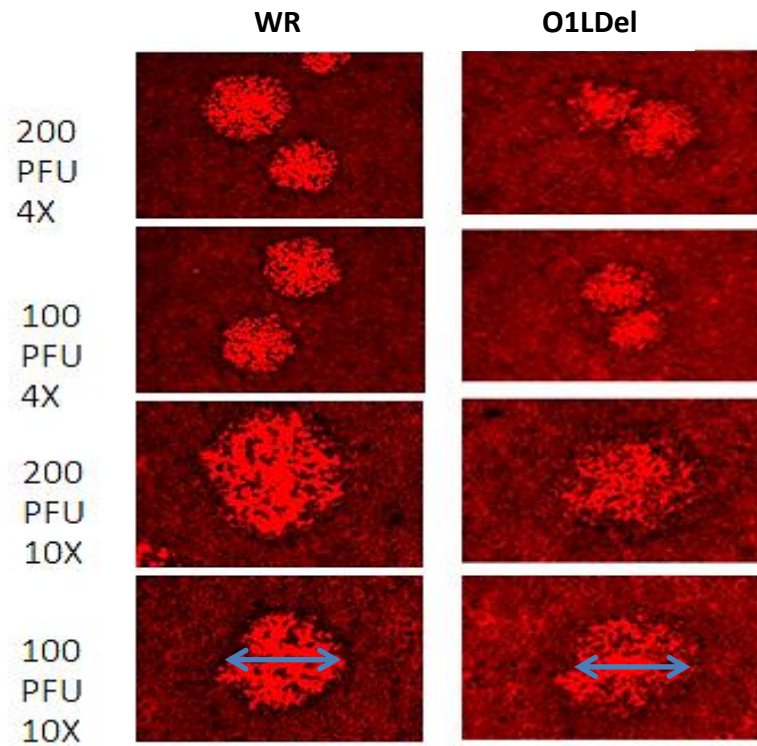


Figure 20. Higher magnification of VACV plaques. The O1LDeI plaques, which initially appeared smaller than WR plaques, have similar diameters to WR plaques upon higher magnification. However, there is less cell clearance in the center of the O1LDeI plaques.

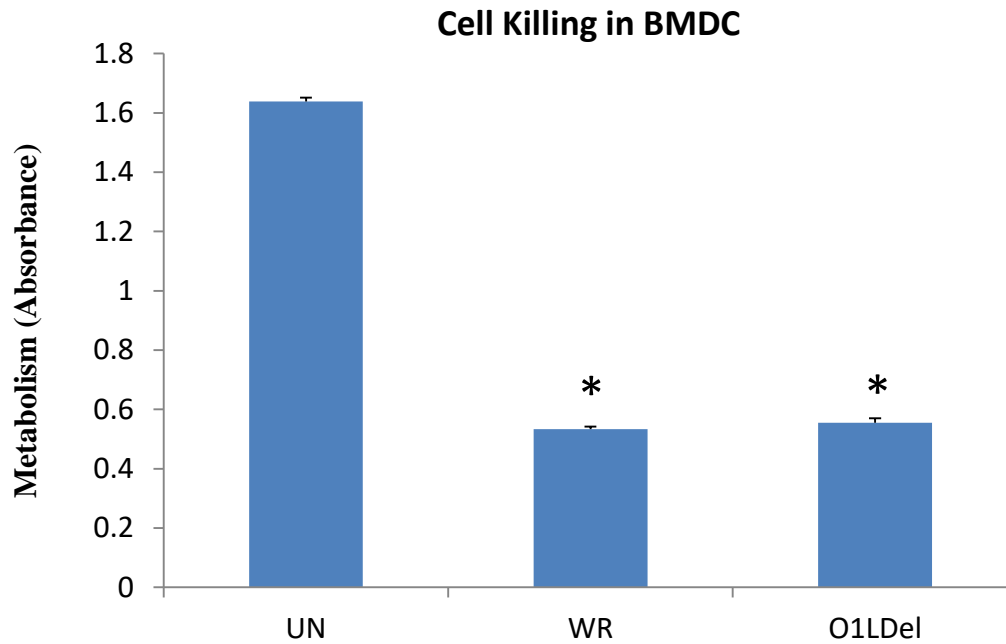
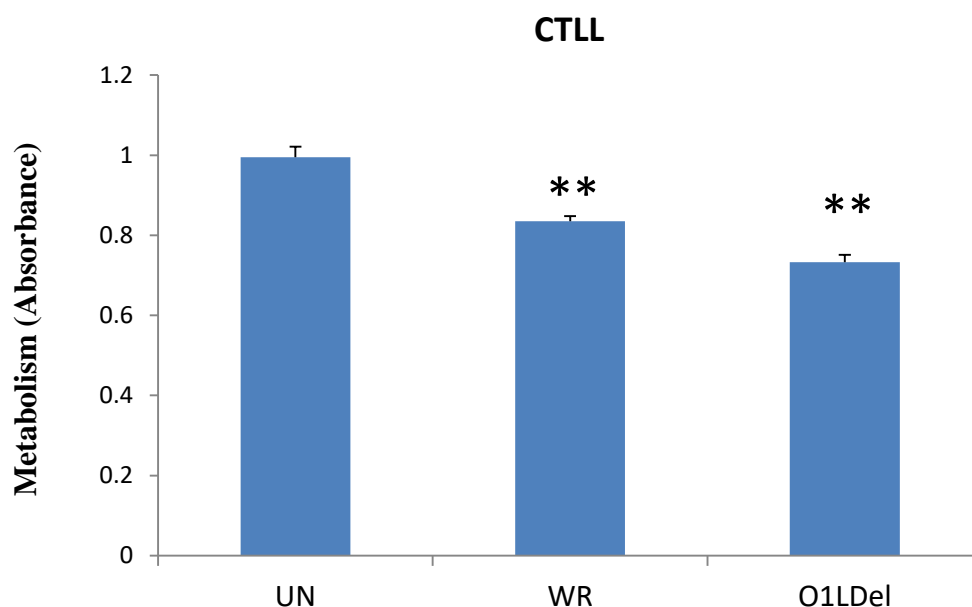


Figure 21. VACV cell killing in BMDC. BMDC were either UN or infected with virus (WR or O1LDe1) in triplicate for approximately 48 h, followed by an MTS assay to measure cell metabolism. A one way ANOVA was performed along with post hoc unpaired two-tailed Student's T-Tests to analyze statistical differences between groups. Data represent the averages (\pm SEM). * $p < 0.0001$, compared to UN.

A)



B)

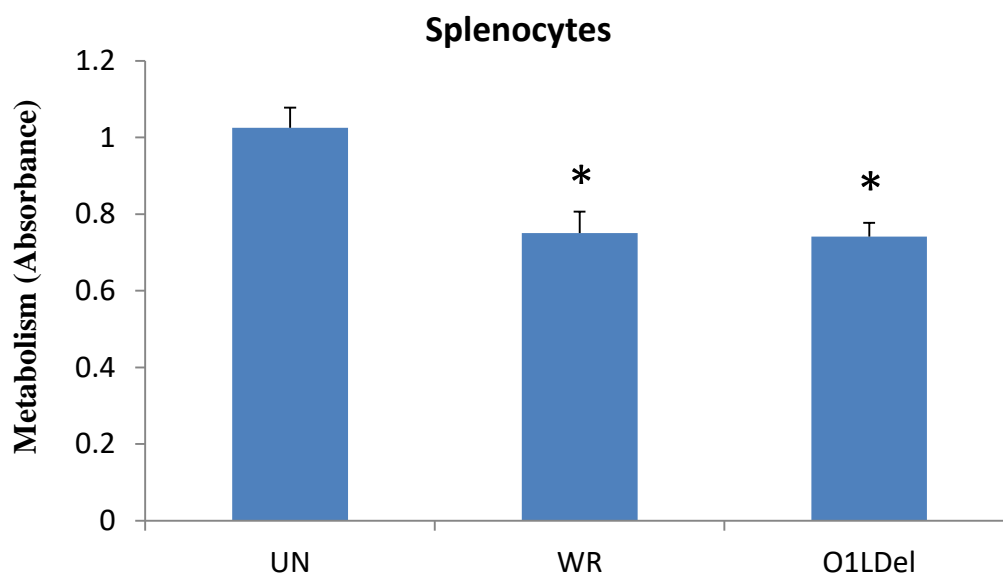


Figure 22. VACV cell killing in CTLL and splenocytes. A) CTLL or B) splenocytes were UN or infected with virus (WR or O1LDeI) for ~48 h, and MTS assays were performed to measure cell metabolism. CTLL data represent the averages of 2 experiments (\pm SEM), and splenocyte data represent the averages of 5 wells (\pm SEM). One way ANOVA and post hoc unpaired two-tailed Student's T-Tests were used for statistical analysis. * $p < 0.01$, ** $p < 0.001$, compared to UN. Metabolism for WR infected CTLL is significantly higher ($p < 0.01$) than O1LDeI infected cells.

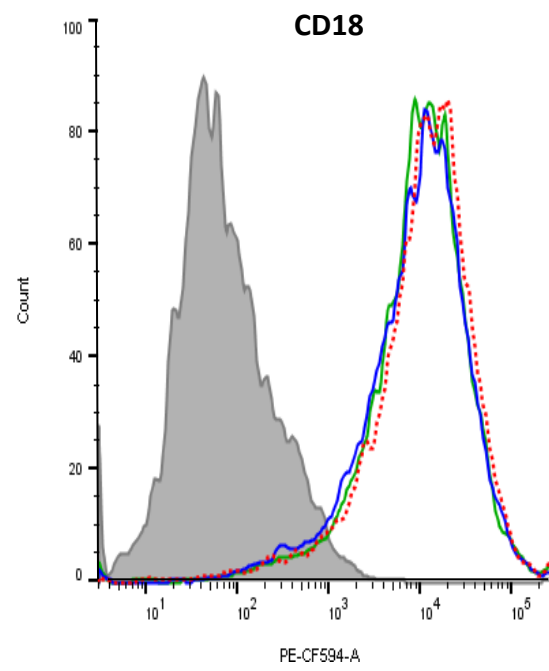
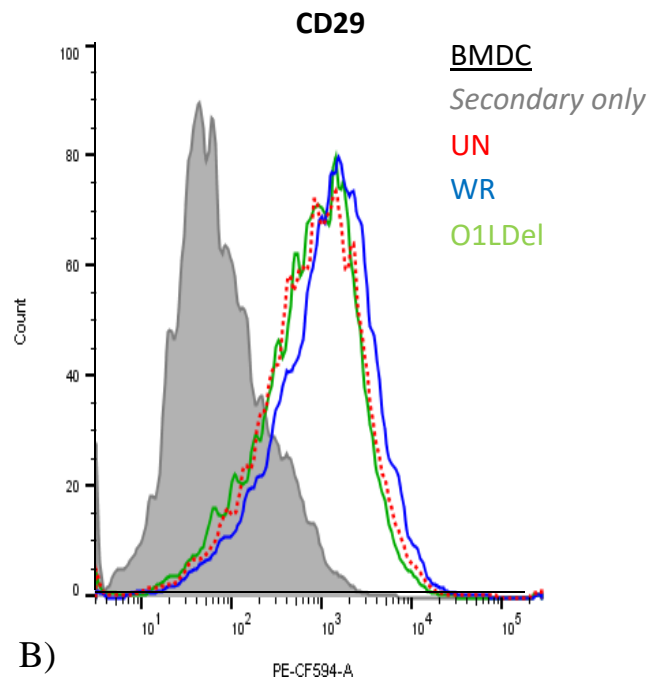
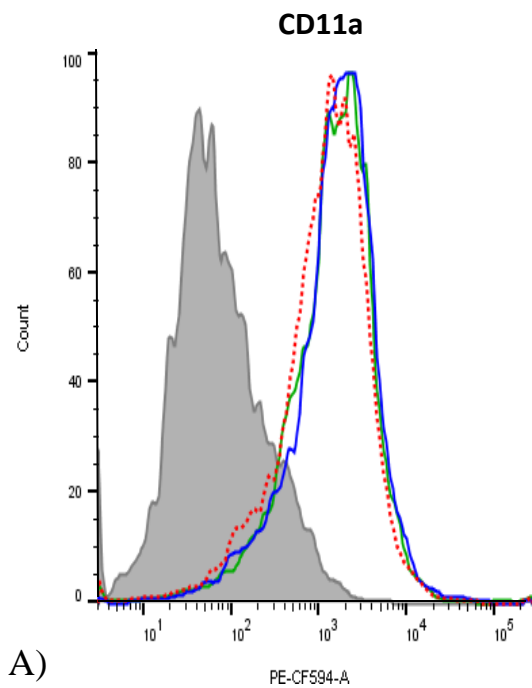


Figure 23. Integrin expression on VACV infected BMDC. BMDC were UN or infected with WR or O1LDeI O/N and stained with primary antibody for A) CD11a (α L), B) CD29 (β 1), and C) CD18 (β 2) integrins. Protein expression was detected using a secondary antibody and measured via flow cytometry. Secondary only stained BMDC were used as a negative control. PE-CF594-A represents the fluorochrome channel used on the FACS Aria to detect antibody signal.

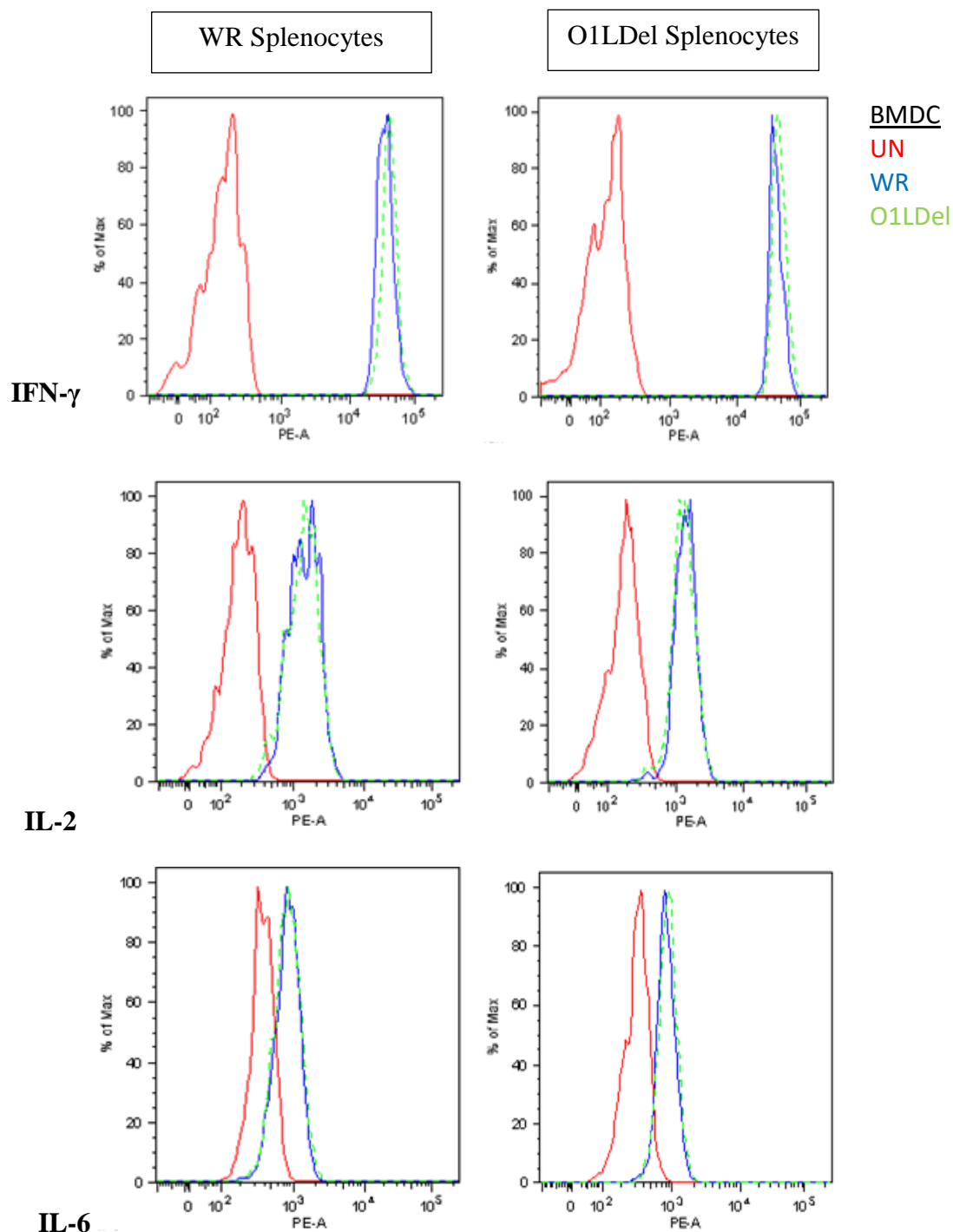


Figure 24. Antigen presentation induced cytokine production. A cytokine bead kit was utilized to test ~ 48 h supernatant samples from antigen presentation assays (splenocytes from 3 week infected mice + BMDC). Production of 7 cytokines was measured including IFN- γ , IL-2, and IL-6, as well as IL-4, IL-10, TNF, and IL-17A (not pictured). Minimal or no IL-4, IL-10, TNF, and IL-17A were detected. Samples were tested in duplicate with similar results. Supernatant samples from week 4 antigen presentation assays were also tested and comparable to week 3 results (data not shown).

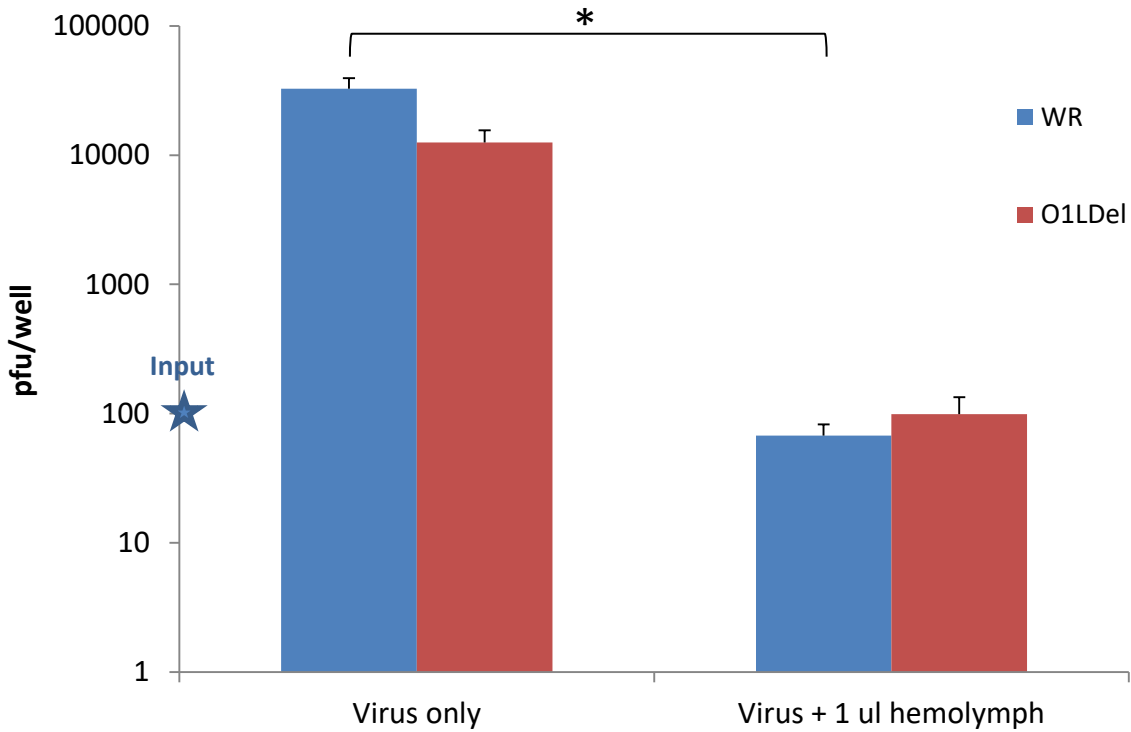


Figure 25. Worm hemolymph and VACV infection. 1 ul of worm hemolymph was incubated with 5.5-6 ul of virus (100 pfu/well) for 10 min. Then, confluent BS-C-1 monolayers in a 96 well plate were infected with the virus/hemolymph mixture for ~21 h. Samples were freeze/thawed 3 times, and the titers were measured by performing viral plaque assays. Data represents the average of 2-3 assays (\pm SEM) and were analyzed using a two-way ANOVA and one-way ANOVA, followed by post hoc unpaired two-tailed Student's T-Tests. * $p < 0.05$.

CHAPTER 5: DISCUSSION

Poxvirus research is important for understanding viral pathogenesis and the identification of virulence genes and their mechanisms. The Roper laboratory previously identified A35 as a virulence gene that inhibits MHC class II antigen presentation (Rehm, Connor et al., 2010). We continued to investigate the function of A35 in the virus life cycle using a new antigen presentation model, which utilized primary murine BMDC as the APC and virus specific CD4⁺ T cells as responders. To begin with, VACV cell killing and replication were analyzed in BMDC to determine if A35 acts by killing APC or altering replication ability. The results did not reveal any A35 effect on the ability of VACV to reduce metabolism or replication in BMDC (Figures 1-2). In fact, BMDC did not appear to be permissive to VACV infection as the number of infectious virus particles produced never exceeded the amount of input virus at all. Pfu/well were similar in the cell lysate and supernatant samples from WR or A35Del infected BMDC over time, suggesting that A35 had no effect. Western blots done by the Roper laboratory have shown that A35 is expressed in infected BMDC, indicating that the virus is able to enter the cells and express viral proteins but not produce infectious virus particles.

To look at the effects of A35 on antigen presentation, antigen presentation assays were performed using infected BMDC (WR or A35Del) presenting viral antigen to CD4⁺ T cells harvested from previously infected mice. T lymphocytes from A35Del infected mice were re-stimulated to produce significantly more ($p < 0.05$) IL-2 relative to T lymphocytes from WR infected mice (Figure 3). These results indicate that A35 inhibits CD4⁺ T cell response *in vivo*, and furthers previously published research from our lab that A35 decreases the number of IFN- γ secreting virus specific T lymphocytes and development of anti-viral cytotoxic T lymphocyte responses in infected mice (Rehm, Jones et al., 2010, Rehm & Roper, 2011).

While there was a difference in T lymphocyte responses between mice infected with A35Del or wild type WR, there was not a difference in IL-2 production between T cells re-stimulated with BMDC infected with WR versus A35Del, suggesting that A35 does not inhibit recall antigen presentation *in vitro* in this model. With the previous model, the Roper laboratory used infected rat PEC (elicited by injection of *Propionibacterium acnes* into the peritoneum 3 days prior) as the APC to present antigen to RSL.11 T cell lines and determined that A35 acts in the APC to inhibit antigen presentation *in vitro* (Rehm, Connor et al., 2010). This difference may be due to the APC type, activation state, or responding T cell types.

Since the antigen presentation assays showed A35 inhibits T lymphocyte response *in vivo*, we wanted to see if A35 inhibits anti-viral antibody production as well. Activated CD4⁺ T cells, specifically T helper type 2, are involved in the immunological cascade by secreting cytokines like IL-4, 5, and 13 that activate B cells to multiply and mature into antibody producing plasma cells (Marshall, Warrington, Watson, & Kim, 2018). Therefore, inhibition of T cell response by A35 may lead to a reduction in anti-viral antibody production. ELISA data revealed that mice infected with the A35Del virus produced more anti-viral antibody compared to WR infected mice with the most significant difference occurring in week 3 infected mice (Figure 5). The antibody used to detect mouse Ig was made in goats immunized with mouse IgG, IgM, and IgA and was shown to detect mouse IgG₁, IgG_{2a}, IgG_{2b}, IgG_{2c}, IgG₃, IgM, and IgA. It was also found that it takes over 50 times more sera from WR infected mice compared to sera from mice infected with A35Del to reach the same absorbance (Figure 6). Collectively, this data indicates that A35 inhibits anti-viral antibody production, which corresponds with data published by the Roper lab (Rehm, Jones et al., 2010). The Roper lab has also examined the effects of A35 on isotype switching and found mice infected with an A35 deletion mutant of

MVA produced significantly more VACV-specific IgG, IgG₁, and IgG_{2a} compared to wild type, and there was no significant difference in IgM production (Rehm & Roper, 2011). This suggests A35 in MVA inhibits isotype switching to IgG and subclasses IgG₁ and IgG_{2a}. In addition to antibody production, it has been shown A35 in VACV also affects spleen size and cellularity, CTL response, and the number of VACV specific IFN- γ secreting cells that develop in mice (Rehm, Jones et al., 2010). These results support that the inhibition of antigen presentation by A35 culminates in the reduction of downstream anti-viral responses.

To this point, A35 has been shown to be immunoregulatory in mice; however the mechanism is still unknown. The Roper laboratory previously measured MHC class II and CD86 expression on APC and found A35 caused little to no reduction in their expression (Rehm, Connor et al., 2010). We decided to look at the effect of A35 on integrins, which are proteins also involved in the APC/T cell interaction and formation of the immunological synapse. If A35 were to inhibit integrin expression and impede formation of the synapse, it may result in reduced activation of CD4⁺ T lymphocytes. We measured CD11a, CD18, and CD29 integrin expression on infected cells and discovered that while the three integrins were detected on BMDC, A35 does not appear to inhibit their expression. Integrin levels were similar between UN, WR, and A35Del BMDC (Figure 7).

Another possible mechanism involves endosomes. Previous studies by the Roper laboratory have shown A35 localizes to endosomes in PEC and disrupts presentation of antigenic peptides in the cleft of MHC class II on the surface of APC (Rehm, Connor et al., 2010). A35 partially colocalized with endosomal markers for early and late endosomes as well as lysosomes and multivesicular bodies in PEC, indicating that A35 associates with a subset of endosomes and may be acting on endosomal processes. To inhibit antigen presentation, A35 could be interfering

with endosomal uptake, trafficking, or maturation so that viral antigens are not processed normally. An additional point of inhibition may be at the exchange of CLIP peptide for antigen in the MHC class II binding groove. When MHC class II molecules are synthesized in the ER, they complex with a protein called the invariant chain, which keeps the MHC class II molecule in the right conformation and blocks other proteins from interacting with the binding groove. The invariant chain is then cleaved leaving the CLIP peptide, which is exchanged for antigen by the human leukocyte antigen DM protein (HLA-DM). A35 may be interfering with the ability of HLA-DM to remove CLIP and load the viral antigenic peptide. This potential mechanism would account for the increased surface expression of CLIP peptide and the reduction of an endogenous peptide in MHC class II detectable on the surface of VACV infected cells when A35 is present (Rehm, Connor et al., 2010). Current studies in the Roper laboratory are focused on determining what proteins A35 interacts with and how A35 affects endosomes to further investigate its mechanism.

While researching A35, the Roper laboratory discovered that the A35Del virus was still able to block antigen presentation in the PEC model, suggesting there are multiple poxvirus proteins that function individually to decrease antigen presentation (Rehm, Connor et al., 2010). We examined the function of another poxvirus virulence gene, O1L, which was identified with A35 to be conserved in mammalian tropic poxviruses and unique to poxviruses, and there was no evidence to suggest O1L was required for replication. Studies by the Roper laboratory have indicated O1L may play an important role in poxvirus virulence in mice, and they hypothesized that it is an immunoregulatory gene/protein. A previous student in the Roper laboratory reported that O1L decreased antigen presentation. To further look at the effects of O1L on antigen presentation, antigen presentation assays were performed with the BMDC model. We found no

significant difference in T lymphocyte response between T cells from O1L Δ el and WR infected mice (Figure 8). The assays were repeated under multiple conditions that encompassed various mouse strains, mouse infection times, cell numbers, antigens and antigen concentrations, and supernatant time points. Antigen presentation assays were performed with a titration of BMDC including 10,000, 50,000, and 100,000 cells (Figures 8-11A), as well as BMDC infected with an MOI of 0.5 (Figures 12, 14A), 1 and 100 (Figure 11B), which allowed us to look at T cell responses when re-stimulated with different availabilities of viral peptides and BMDC. With a higher MOI, as virus is added, it is expected that the number of viable BMDC able to present antigen would decrease due to viral infection. We saw when examining VACV cell killing in BMDC that VACV does reduce cellular metabolism (Figure 1, Figure 21). Overall, there was no evidence that O1L inhibits antigen presentation or T cell response based on the data collected (Figures 8-14).

Next, we wanted to examine if O1L interferes with anti-viral antibody production, perhaps by inhibiting a cytokine necessary for B cell activation, cell interactions, or targeting the B cell itself. ELISA data showed there were similar amounts of anti-viral antibody produced between WR and O1L Δ el infected mice for 1 to 4 weeks (Figure 15). Repeated experiments in 2 different mouse strains at multiple time points also revealed no significant difference in anti-viral antibody production in mice infected with WR and O1L Δ el viruses (Figures 16-18). These results suggest that O1L in VACV does not appear to inhibit anti-viral antibody production.

Based on the data collected, O1L does not appear to inhibit anti-viral antibody, antigen presentation or T cell response, but studies by the Roper laboratory and others (Schweneker et al., 2012) have shown it is important in virulence. Another possible function of O1L is that it promotes viral virulence by sustained activation of the Raf/MEK/ERK pathway (Schweneker et

al., 2012). This pathway activates transcription factors that influence a variety of processes including cell motility, differentiation, survival and death, and growth (Shaul & Seger, 2007; Vial & Pouyssegur, 2004; Yoon & Seger, 2006). A number of viruses activate this pathway to promote a metabolically active cellular environment to support viral replication (Schweneker et al., 2012). Viral plaque assays were performed to look at replication and spread between O1LDel and WR viruses, and we found that for the majority of the time the plaques appeared similar between the two viruses (Figure 19). However, in some cases O1LDel plaques appeared smaller than wild type, which was associated with less cell clearance in the center of O1LDel plaques while the diameter of the plaques was the same (Figure 20). A potential explanation for the reduced cell clearance is that O1L affects cell killing, possibly through the role of the Raf/MEK/ERK pathway in cell survival and death. However, MTS assays in BMDC, splenocytes, and CTLL revealed that WR and O1LDel viruses reduced cellular metabolism in a similar manner (Figures 21-22).

We next looked at the potential effects of O1L on cell migration. A previous student in the Roper laboratory found that there were differences in cell migration between BS-C-1 cells infected with O1LDel compared to wild type WR infected cells. To investigate this finding, expression of CD11a, CD18, and CD29 integrins, which are important for leukocyte migration to areas of infection in the body, were measured on BMDC. While the three integrins were detected on the BMDC, there was not a difference in expression between UN, WR, or O1LDel infected cells (Figure 23), providing no evidence that O1L affects cell migration through this mechanism. I repeated the scratch assays performed previously in the Roper laboratory to determine if there was a reproducible difference in migration between WR and O1LDel infected cells and did not find any difference (data not shown). Another option is to perform an

alternative assay such as a transwell cell migration assay. This assay involves adding cells on top of a transwell insert, followed by the addition of a chemoattractant to the lower chamber, and then the number of migrated cells is quantified (Justus, Leffler, Ruiz-Echevarria, & Yang, 2014).

An additional cellular function related to the Raf/MEK/ERK pathway is differentiation, which may affect cytokine production. Although O1L did not appear to affect IL-2 production in relation to antigen presentation, it was possible it affects the production of other antigen presentation induced cytokines. A cytokine bead assay was used to look at the production of important Th1/Th2/Th17 cytokines in antigen presentation supernatants, and there was no evidence to indicate O1L affects their production (Figure 24). Perhaps O1L acts downstream of cytokine production and interferes with the signal transduction in the target cells. Further experiments should focus on determining with what proteins O1L interacts.

To continue to investigate the function of O1L in the virus life cycle, we hypothesized that if there was no evidence to suggest O1L inhibits the adaptive immune response, it may instead affect the innate immune system. While the adaptive immune response is crucial for protection against VACV (Wyatt et al., 2004), the innate immune response is the first line of defense during an infection. Studies have shown that VACV encodes numerous proteins that interfere with innate immune mechanisms such as complement, interferons, and NF- κ B activation (Smith et al., 2013). To study if O1L has a regulatory role in the innate immune response, hemolymph from waxworms was utilized. *Galleria mellonella* larvae, also known as waxworms are an inexpensive and simple way to study immune responses to pathogens (Pereira et al., 2018). They have many structural and functional similarities to the innate immune response in mammals including immune cells called hemocytes which have similar phagocytic abilities to neutrophils as well as humoral components such as complement like proteins and

reactive oxygen species (Pereira et al., 2018). Varying concentrations of hemolymph were incubated with virus (WR or O1L~~Del~~) and added to BS-C-1 cells. Alternatively, BS-C-1 cells were pre-incubated with hemolymph and infected with virus. Then, the number of pfu/well were measured via viral plaque assays. The addition of hemolymph to virus resulted in a large reduction in pfu compared to cells infected with virus only (Figure 25), showing that the hemolymph somehow affected VACV infection and/or replication or cell killing. Virus incubated with increasing amounts of hemolymph or cells that were pre-incubated with hemolymph before viral infection showed an even greater reduction in plaque numbers (data not shown). Additional assays are needed to determine exactly how the hemolymph reduces pfu.

O1L~~Del~~ + hemolymph infected cells produced a similar amount of infectious virus particles compared to WR + hemolymph infected cells, providing no evidence that O1L inhibits the innate immune response in this model. If O1L were affecting some component in the hemolymph to promote viral infection, it would be expected that the WR + hemolymph infected cells would have greater pfu compared to the O1L~~Del~~ + hemolymph infected cells because with O1L present, the virus would be able to inhibit the anti-viral effects of the hemolymph.

Although the preliminary results provide no evidence that O1L affects the innate immune response, it would be beneficial to repeat the worm hemolymph experiments to determine if an O1L phenotype might be observed under optimized conditions. Future experiments should include less hemolymph added because even 1 ul was able to greatly reduce pfu. By using a smaller amount of hemolymph, we may be able to see more differences between WR and O1L~~Del~~. The hemolymph could also be spun down to remove debris as well as separate out the cellular components. In this way, we would be able to examine the effects of the humoral component of the hemolymph on VACV infection, which could provide data on how the

hemolymph is reducing pfu in VACV infected cells. Since O1L has been shown to be conserved in mammalian tropic poxviruses, the innate immune response in mice could be examined by performing IFN alpha and IFN beta ELISPOT experiments with splenocytes from O1L~~Del~~ and WR infected mice. Another potential future experiment is an assay to look at the ability of important innate immune cells like natural killer cells to lyse target cells, using cells from WR or O1L~~Del~~ infected mice. Though the function of O1L remains unclear, there are several avenues that appear promising for future research including the potential impact of O1L on the innate immune response.

While there is still work to be done to elucidate the mechanisms of A35 and O1L in the VACV life cycle, their characterization as virulence genes suggests their removal from vaccine strains may improve the safety and efficacy of smallpox vaccines and poxvirus vectors. The Roper laboratory has performed lethal challenge models using both A35~~Del~~ and O1L~~Del~~ viruses by vaccinating mice with one of the deletion mutant viruses, followed by infection with a lethal dose of WR (Rehm, Jones et al., 2010). In both cases, mice vaccinated by a single dose of A35~~Del~~ or O1L~~Del~~ were protected against the lethal secondary infection and maintained their weight (Rehm, Jones et al., 2010). Another laboratory used the Lister vaccine strain of VACV, which is utilized for vaccination in Russia, to create a recombinant virus with multiple targeted inactivations of virulence genes including A35 that also proved to be highly immunogenic (Yakubitskyi, Kolosova, Maksyutov, & Shchelkunov, 2016). This approach is promising in that it strives to generate highly attenuated vaccines that are still replication competent and able to initiate a strong immune response. Understanding how poxviruses turn off immune responses will aid in anti-viral drug design, improve vaccines, and may allow us to mimic poxvirus immunosuppression to control autoimmune diseases.

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APPENDIX - Animal Use Protocol Letter



Animal Care and
Use Committee
212 Ed Warren Life
Sciences Building
East Carolina University
Greenville, NC 27834-4354

252-744-2436 office
252-744-2355 fax

August 9, 2017

Rachel Roper, Ph.D.
Department of Micro/Immuno
Brody 5E-106
East Carolina University

Dear Dr. Roper:

Your Animal Use Protocol entitled, "Analysis of Poxvirus Virulence and Improved Vaccines Using Murine Vaccination and Challenge Models" (AUP #K173) was reviewed by this institution's Animal Care and Use Committee on August 9, 2017. The following action was taken by the Committee:

"Approved as submitted"

Please contact Aaron Hinkle at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

Administrative approval *JP*
East Carolina University Animal Use Protocol (AUP) Amendment Form
 Latest Revision, March 2016 *11/17/17*

FOR IACUC USE ONLY	
AUP # <i>K173</i>	
Date received: <i>11/16/17</i>	
Full Review and date:	Designated Reviewer and date:
Approval date: <i>11/17/17</i>	
Pain Category: <i>D</i>	
Amendments approved:	
Minor Amendment: <i>1 admin</i>	
Significant Amendment:	If so, number?

Please fill out completely and email to davenportp@ecu.edu or iacuc@ecu.edu

PROJECT INFORMATION: Please list AUP Number and Title

K173 Analysis of Poxvirus Virulence and Improved Vaccines using Murine Vaccination and Challenge Models

Principal Investigator:

Dr. Rachel Roper

1. Please explain in simple, non-technical language, the purpose or rationale for the protocol amendment.

We are adding new personnel, Alexandra Hayes a MS student in Brody. She has completed DCM animal handling training. There are no changes in the AUP other than a new person working on the project

2. Will different people use the animals or are new personnel being added to the AUP? Will previously approved personnel be assuming new roles or responsibilities?

10/5/17
 If so, list qualifications and training.

We are adding new personnel, Alexandra Hayes. She has completed all required training including the DCM animal handling training (10/05/17) and has been trained by Gwen (Bass) Jones on PPE and entry/exit in the animal room. She will be trained and supervised by Gwen or myself in any procedures that are new to her. Gwen has experience with all techniques in the AUP and 10 years of experience.

3. Have protocol related hazards (transgenic animals, infectious, chemical or biologic agents) changed with this amendment? *Choose an item* **NO**

If so, please describe the hazard and the oversight committee associated with this hazard (see AUP form II.C.1). If any hazardous agents have changed since the original AUP, please fill out the attached Hazardous Agents Form (Appendix 1). Oversight committee approval is required before the amendment can be approved by IACUC. No changes in AUP other than new person working on the project

4. Please indicate changes to the animals or animal numbers by addressing them in parts a, b, and/or c. No changes in AUP other than new person working on the project

a. Will the strain or sex of the animals change? *Choose an item*

If so, please list changes (use complete strain nomenclature and a brief description of the line/strain when possible). No changes

b. Will the phenotype of mutant, transgenic or knockout animals predispose them to any health, behavioral, physical abnormalities, or cause debilitation effects in experimental manipulations? (if yes, describe)

No changes

c. Will additional animals be needed? Choose an item

If so, please justify the increase in animal numbers, using statistical justification whenever possible.

No changes

5. Are there any unusual husbandry and environmental conditions required, including single housing? Social housing is now the default housing for social species.

No changes

If yes, then describe conditions and justify the exceptions to standard housing. (see AUP form III.C.5-7)

No changes

6. Will there be new non-surgical procedures involving the animals? Choose an item

Please describe these procedural changes with the same level of detail as is required in a new protocol (see AUP form IV. B-R). Addition of or changes to surgical procedures are addressed in question 12.

No changes

7. Will additional sampling of body fluids or tissues collections occur? Choose an item

Will additional administration of substances occur? Choose an item

If so, please fill out table(s) below and address non-pharmaceutical grade substances in questions 'a' and 'b' below the table: No changes

Blood or Body Fluid Collection

	Location on animal	Needle/catheter size	Volume collected	Frequency of procedure	Time interval between collections
Blood Collection	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text
Body Fluid Collection	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text
Other	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text

Injections, Gavage, & Other Substance Administration

	Compound	Location & Route of admin	Needle/catheter/gavage size	Max volume admin	Freq of admin (i.e. two times per day)	Number of days admin (i.e. for 5)	Max dosages (mg/kg)
--	----------	---------------------------	-----------------------------	------------------	--	-----------------------------------	---------------------

						days)	
Injection/ Infusion	Click here to enter text.	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text.	Click here to enter text	Click here to enter text
Gavage	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text
Other	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text	Click here to enter text

Pharmaceutical grade drugs, biologics, reagents, and compounds are defined as agents approved by the Food and Drug Administration (FDA) or for which a chemical purity standard has been written/established by any recognized pharmacopeia such as USP, NF, BP, etc. These standards are used by manufacturers to help ensure that the products are of the appropriate chemical purity and quality, in the appropriate solution or compound, to ensure stability, safety, and efficacy. For all injections and infusions for CLINICAL USE, PHARMACEUTICAL GRADE compounds must be used whenever possible. Pharmaceutical grade injections and infusions for research test articles are preferred when available. If pharmaceutical grade compounds are not available and non-pharmaceutical grade agents must be used, then the following information is necessary:

- Please provide a scientific justification for the use of ALL non-pharmaceutical grade compounds. This may include pharmaceutical-grade compound(s) that are not available in the appropriate concentration or formulation, or the appropriate vehicle control is unavailable.
- Indicate the method of preparation, addressing items such as purity, sterility, pH, osmolality, pyrogenicity, adverse reactions, etc. (please refer to ECU IACUC guidelines for non-pharmaceutical grade compound use), labeling (i.e. preparation and use-by dates), administration and storage of each formulation that maintains stability and quality/sterility of the compound(s).

No changes

8. Are animals expected to experience more clinical illness, pain, or distress as a result of the procedures proposed in this amendment? Choose an item

If yes, please complete literature search for alternatives to the use of potentially painful/distressful procedures (see below).

No changes

How will any pain or distress from this procedure be minimized?

No changes

- Include a literature search to ensure that alternatives to all procedures that may cause more than momentary or slight pain or distress to the animals have been considered.

1. Please list all of the potentially painful or distressful procedures in the protocol:

No changes in AUP other than new person working on the project

2. For the procedures listed above, provide the following information (please do not submit search results but retain them for your records):

Date Search was performed:	Click here to enter text
Database(s) searched:	Click here to enter text
Time period covered by the search (i.e. 1975-2013):	Click here to enter text
Search strategy (including scientifically relevant terminology):	Click here to enter text
Other sources consulted:	Click here to enter text

3. In a few sentences, please provide a brief narrative indicating the results of the search(es) to determine the availability of alternatives and explain why these alternatives were not chosen. Also, please address the 3 Rs of refinement, reduction, and replacement in your response. Refinement refers to modification of husbandry or experimental procedures to enhance animal well-being and minimize or eliminate pain and distress. Replacement refers to absolute (i.e. replacing animals with an inanimate system) or relative (i.e. using less sentient species) replacement. Reduction involves strategies such as experimental design analysis, application of newer technologies, use of appropriate statistical methods, etc., to use the fewest animals or maximize information without increasing animal pain or distress.

No changes

9. Will there be addition of or change in the method of anesthesia, analgesia, or euthanasia? [Click here to enter text](#)

If so, describe in detail (see AUP form IV. B, S, and V.).

No changes

10. Will prolonged restraint of conscious animals be required? [Click here to enter text](#)

If so, describe in detail (see AUP form IV. F).

No changes

11. Will surgical procedures be added or surgical plans from the original protocol change? (major, minor, multiple survival, additional procedures). [Click here to enter text](#)

If so, describe in detail (see AUP form IV.S).

No changes

PI Signature: *Rashid L. Rogers* *email* Date: 11/16/17
Oct 31, 2017

Veterinarian: _____ Date: _____

IACUC Chair: _____ Date: _____

administrative approval
11/17/17

